AD_____

Award Number: DAMD17-02-1-0174

TITLE: Neuregulins, Neuroprotection and Parkinson's Disease

PRINCIPAL INVESTIGATOR: David M.Yurek, Ph.D.
Kim B. Seroogy, Ph.D.

CONTRACTING ORGANIZATION: University of Kentucky Research Foundation
Lexington, Kentucky   40506-0057

REPORT DATE: September 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-09-2006	Final	19 Nov 2001 – 30 Aug 2006

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Neuregulins, Neuroprotection and Parkinson's Disease	5b. GRANT NUMBER
	DAMD17-02-1-0174
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
David M.Yurek, Ph.D. and Kim B. Seroogy, Ph.D.	5e. TASK NUMBER
E-Mail: David.Yurek@uky.edu	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
University of Kentucky Research Foundation Lexington, Kentucky 40506-0057	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The main hypothesis being tested in this research project is that neuregulins, primarily focusing on glial growth factor-2 (GGF2), is neuroprotective and/or neurorestorative for the damaged dopaminergic nigrostriatal system. Other hypotheses to be tested are that GGF2 augments functional indices of the dopaminergic nigrostriatal system and that one of the intracellular signaling mechanisms mediating the protective effects of neuregulins for dopamine neurons involves the PI3-kinase pathway. These studies are being conducted in normal rats, in a 6-OHDA rat model of Parkinson's disease, in primary neuronal cultures of midbrain dopamine cells, and in a dopaminergic cell line. Overall, results from these studies may form the basis for the therapeutic application of neuregulins to the treatment of neurotoxin-induced neurodegenerative disorders such as Parkinson's disease.

15. SUBJECT TERMS
neurotrophic factor, neuroprotection, Parkinson's disease, nigrostriatal pathway, rodent, dopamine, striatum, substantia nigra, cell culture, erbB4, GGF2

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	37	19b. TELEPHONE NUMBER *(include area code)*
U	U	U			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION

The main hypothesis being tested in this research project is that neuregulins, primarily focusing on glial growth factor-2 (GGF2), is neuroprotective and/or neurorestorative for the damaged dopaminergic nigrostriatal system. Other hypotheses to be tested are that GGF2 augments functional indices of the dopaminergic nigrostriatal system and that one of the intracellular signaling mechanisms mediating the protective effects of neuregulins for dopamine neurons involves the PI3-kinase pathway. These studies are being conducted in normal rats, in a 6-OHDA rat model of Parkinson's disease, in primary neuronal cultures of midbrain dopamine cells, and in a dopaminergic cell line. Overall, results from these studies may form the basis for the therapeutic application of neuregulins to the treatment of neurotoxin-induced neurodegenerative disorders such as Parkinson's disease.

BODY

Experiment #1a,b,c: These experiments were designed to determine if infusion of neuregulin augments nigrostriatal dopamine function in the rat. In vivo microdialysis was used to examine the acute effects of neuregulin on basal dopamine release in the nigrostriatal system. Our studies show that unilateral infusion of the neuregulin heregulin-β1 (HRG-β1; 10 μg) and GGF2 (3 µg) just above the substantia nigra resulted in a rapid increase in dopamine overflow in the ipsilateral striatum. Thus, acute administration of neuregulins can modulate the activity of mesostriatal dopaminergic neurons by enhancing at least one important index of nigrostriatal function. These findings have been published; see Appendix (*Yurek et al., Brain Research 1028: 116-119).*

A logical interpretation of these findings is that the neuregulins ligands injected supranigrally are acting via binding to neuregulin receptors expressed by midbrain dopaminergic neurons to elicit their effects. However, GABAergic neurons are also present in the ventral midbrain and could also contain neuregulin receptors, potentially confounding our interpretation. Therefore, to address this issue, we processed rat brains using double-labeling in situ hybridization for the neuregulin receptor ErbB4 and GAD mRNAs (*Seroogy et al., 2004, 2005 abstracts*). In the ventral mesencephalon, only rare examples of GAD mRNA/ErbB4 mRNA coexistence could be detected in GABAergic neurons of the substantia nigra and ventral tegmental area. In contrast, in the hippocampal formation and cerebral cortex, the vast majority of all GAD mRNA-expressing interneurons also contained ErbB4 mRNA. In the striatum, all of the large, scattered GAD mRNA-positive interneurons were also labeled for ErbB4 mRNA. These results indicate that midbrain GABAergic neurons do not directly bind neuregulin ligands, at least via the ErbB4 receptor, supporting our above hypothesis that only the midbrain dopamine neurons, which are known to express ErbB4, are mediating the actions of the infused neuregulin ligands. In contrast, numerous cortical, hippocampal and striatal GABAergic interneurons, due to their expression of ErbB4, have the capacity to respond to neuregulin family members. These data raise the possibility for neuregulin modulation of forebrain GABAergic interneuron mechanisms. These results are being extended and will be written up for publication in the near future.

Experiment #2a,b: This neuroprotection/neurorestoration study was designed to test if supranigral administration of GGF2 prior to or after neurotoxic (6-hydroxydopamine; 6-OHDA) lesion of the nigrostriatal system protects the nigral dopamine neurons from the neurotoxic damage, as determined both morphologically and functionally. For the neuroprotection study, adult male Sprague Dawley rats received two unilateral injections of 6-OHDA (10 µg each in 2 µl saline + 0.2% ascorbic acid) or 6-OHDA vehicle (saline + ascorbic acid) stereotaxically placed into the right striatum to create the partial lesion model. As described in a previous progress report, we chose the intrastriatal 6-OHDA partial lesion model because the degeneration of dopaminergic neurons in the substantia nigra occurs progressively and a certain percentage of dopamine neurons are spared from the injury. Therefore the tested neuregulin can theoretically exert its bioactivity on both intact and injured dopaminergic neurons in this lesion model. Immediately after 6-OHDA lesioning (i.e. during the same surgery), the animals received continuous supranigral infusion of GGF2 (6 µg/day) or PBS (vehicle). Osmotic minipumps were used for continuous infusion of the neuregulin ligand or vehicle for 1 week. The cannula tip attached to the osmotic pump was stereotaxically placed approximately 1 mm above the right substantia nigra. At 2 weeks and 4 weeks post-lesioning, the rats were tested behaviorally using asymmetrical forelimb use behavior (cylinder test); attenuation of asymmetrical forelimb use was used as a measure of neuregulin-mediated functional recovery. At the end of the 5th post-lesion week, the animals were euthanatized and the brains removed for immunohistochemical analysis of the dopamine cell marker tyrosine hydroxylase (TH) in the substantia nigra. Our results demonstrate that supranigral infusion of GGF2 significantly protects nigral dopamine neurons against 6-OHDA-induced degeneration and significantly improves behavioral functional recovery in this rat model of Parkinson's disease (see Figs. 1-3 in Appendix I). These intriguing findings are currently being written up for publication (*Zhang et al., in preparation*).

For the neurorestoration study, we also evaluated post-treatment of the substantia nigra dopaminergic neurons with GGF2 *after* neurotoxic lesion of the nigrostriatal pathway to determine if the dopaminergic neurons and associated behavioral indices are rescued or restored after degeneration has commenced. The procedure was the same as described above for the neuroprotection experiment, except the minipump implantation was delayed until 2 weeks post-6-OHDA lesion. Following this neuregulin post-treatment paradigm, our subsequent morphological and behavioral analyses did not reveal significant improvement in nigral dopamine cell survival or functional recovery. Although we were disappointed by these results, several explanations are conceivable. First, of course, is that GGF2 may not be effective in dopamine neuronal repair after an insult has already occurred. However, other factors that need to be further evaluated include increasing the dose of GGF2 infused as well as the duration of the infusion period. Also, an alternative infusion site to consider for the neuregulins is the striatum. The fact that a different batch of recombinant human GGF2 was used for this neurorestoration study (prepared by Acorda Therapeutics, Inc.), as opposed to the above neuroprotection study (prepared by CeNeS Pharmaceuticals; firm now defunct), may offer another explanation. In any event, we feel strongly that further studies on the neuroprotective as well as neurorestorative potential of neuregulins for treatment of the injured nigrostriatal system are warranted.

Experiment #3a,b,c: These experiments were designed to determine the protective and trophic capabilities and potential mechanisms of neuregulin treatment against neurodegenerative insults in dopamine cell cultures. Our *in vitro* studies in fetal rat mesencephalic cell cultures have been completed. We found that GGF2 exerts neurotrophic and neuroprotective effects upon normal and injured dopaminergic neurons developing in the serum-free cultures. Specifically, GGF2 significantly promoted dopaminergic neuronal survival, enhanced neurite outgrowth (in terms of both fiber length and number of branches), and increased dopamine uptake. GGF2 also protected the dopamine cells against 6-OHDA-induced degeneration. We demonstrated that ErbB4 mRNA is expressed in rat embryonic day 14 ventral mesencephalic flexure (from which the cultures are derived) in an overlapping pattern with TH mRNA. We showed that three neuregulin receptors (ErbB2, ErbB3, ErbB4) are expressed in the primary midbrain cultures and that the number of astrocytes is minimal. Together, these data strongly suggest that GGF2 is a novel trophic/protective factor for mesencephalic dopaminergic neurons. These comprehensive findings have been published; see Appendix (*Zhang et al., J. Neurochem. 91:1358-1368, 2004*).

To determine the potential mechanism(s) of neuregulin protection of dopaminergic cells against neurodegenerative insults, we collaborated with Drs. Mark Mattson and Wenzhen Duan (Laboratory of Neurosciences, National Insitute of Aging). First, the neuroprotective effects of neuregulin (heregulin-1β in this case) in the human neuroblastoma cell line SK-N-MC have been examined. These cells express tyrosine hydroxylase, produce dopamine, and are vulnerable to degeneration induced by iron or the complex I inhibitor rotenone. Because this homogeneous population is more easily amenable to mechanistic investigations than the fetal midbrain cultures (which contain only ~10-20% dopamine cells), we performed the experiments in this cell line. We first demonstrated the presence of the protein tyrosine kinase neuregulin receptor ErbB4 in the cells (Fig. 4). Neuregulin pretreatment protected the SK-N-MC cells from both oxidative and metabolic insults in both cell survival and mitochondrial dysfunction assays (Figs. 5,6). Inhibition of the PI3 kinase/Akt pathway, one of the 2nd messenger systems known to be activated downstream of ErbB4, completely abrogated the neuroprotection afforded by neuregulin (Figs. 5,6). Moreover, phosphorylated-Akt rapidly increased in the presence of neuregulin in the cultures, an effect that was severely attenuated by administration of the PI3 kinase inhibitor wortmannin (Fig. 7). Thus, these results indicate that neuregulin protects these dopaminergic cells from insults relevant to Parkinson's disease via the PI3K/Akt signaling pathway. These findings are currently being written up for publication (*Seroogy, Duan, Yurek et al., in preparation*).

KEY RESEARCH ACCOMPLISHMENTS

• Neuregulin administration enhances basal release of dopamine in the rat nigrostriatal system in vivo.

• GGF2 infusion promotes the behavioral functional recovery of the injured nigrostriatal system.

• GGF2 infusion protects midbrain dopaminergic neurons against neurotoxin-induced degeneration in vivo.

6

- GGF2 promotes dopaminergic neuronal survival, enhanced neurite outgrowth, and increased dopamine uptake in primary midbrain neuronal cultures.

- GGF2 protects cultured dopamine cells against neurotoxin-induced degeneration.

- Neuregulin protects dopaminergic cells from insults relevant to Parkinson's disease via the PI3K/Akt intracellular signaling pathway.

- Taken together, these results indicate that neuregulins are neurotrophic and neuroprotective factors for nigrostriatal dopaminergic neurons, both in vitro and in vivo.

REPORTABLE OUTCOMES

Manuscripts:

Zhang, L., Fletcher-Turner, A., Lundgren, K.H., Marchionni, M., Yurek, D.M. and Seroogy, K.B. (2004) Neurotrophic and neuroprotective effects of the neuregulin glial growth factor-2 on dopaminergic neurons in rat primary midbrain cultures. *Journal of Neurochemistry* 91: 1358-1368.

Yurek, D.M., Zhang, L., Fletcher-Turner, A. and Seroogy, K.B. (2004) Supranigral injection of neuregulin1-β induces striatal dopamine overflow. *Brain Research* 1028: 116-119.

Seroogy, K.B., Duan, W., Yurek, D.M., Lundgren, K.H., Zhang, L., Gash, D.M., Kornblum, H.I. and Mattson, M.P. Primate dopaminergic neurons express the ErbB4 neuregulin receptor in vivo and are protected from oxidative and metabolic insults by neuregulin in vitro. *In preparation.*

Zhang, L., Yurek, D.M., Lundgren, K.H., Marchionni, M., Dickerson, J., Hemmerle, A. and Seroogy, K.B. Infusion of the neuregulin GGF2 promotes functional recovery and enhances survival of midbrain dopaminergic neurons in the injured rat nigrostriatal system. *In preparation.*

Book Chapter:

Seroogy, K.B. and Zhang, L. (2006) Neuregulins. In: Handbook of Biologically Active Peptides, A.J. Kastin, (ed.), Elsevier Academic Press, Amsterdam, pp. 1401-1407.

Abstracts:

Zhang, L., Fletcher-Turner, A., Lundgren, K.H., Cheng, L., Marchionni, M.A., Yurek, D.M. and Seroogy, K.B. (2002) The long-term survival of cultured dopaminergic neurons is promoted by the neuregulin GGF2. *Soc. Neurosci. Abstr.* 28: #427.6.

Zhang, L., Apparsundaram, S., Marchionni, M., Yurek, D.M. and Seroogy, K.B. (2003) The neuregulin GGF2 promotes fetal dopaminergic neuronal survival in rat primary midbrain cultures. *10th Annual American Society for Neural Transplantation Meeting*, Clearwater Beach, FL. *Exp. Neurol.* 181: 111-112.

Zhang, L., Yi, A., Fletcher-Turner, A., Marchionni, M.A., Yurek, D.M. and Seroogy, K.B. (2003) The neuregulin GGF2 enhances dopaminergic neuron survival in rat primary midbrain cultures and promotes functional recovery in rats with partial 6-OHDA lesions. *Soc. Neurosci. Abst.* #787.2.

Seroogy, K.B., Herman, J.P. and Lundgren, K.H. (2004) ErbB4 neuregulin receptor and GAD mRNAs are extensively colocalized in forebrain, but not ventral midbrain, GABAergic neurons. *Soc. Neurosci. Abst.* #38.5.

Seroogy, K.B., Zhang, L., Marchionni, M.A. and Yurek, D.M. (2005) Neuroprotective and neurotrophic actions of neuregulin polypeptides in the dopaminergic nigrostriatal system. *2004 Summer Neuropeptide Conference*, Miami Beach, FL. *Neuropeptides.* 39: 43.

Seroogy, K.B., Herman, J.P. and Lundgren, K.H. (2005) Numerous forebrain, but not ventral midbrain, GABAergic neurons express the neuregulin receptor ErbB4. *12th Annual American Society for Neural Transplantation Meeting*, Clearwater Beach, FL. *Exp. Neurol.* 193: 259.

Presentations (K.B. Seroogy, Ph.D.):

"Neuroprotection of Dopamine Cells by Neuregulins in Models of Parkinson's Disease"
(Invited Seminar, Dept. of Neurology, University of Cincinnati, Cincinnati, OH, April 15, 2002)

"Neuregulins and Parkinson's Disease: Pre-Clinical Studies"
(Grand Rounds, Dept. of Neurology, University of Cincinnati, Cincinnati, OH, October 22, 2003)

"Neuroprotective and Neurotrophic Effects of Neuregulins on Dopamine Cells Both In Vitro and In Vivo" (Invited Seminar, Acorda Therapeutics, Inc., Hawthorne, NY, December 9, 2003)

"Neuregulin Growth Factors and Parkinson's Disease"
(Co-Keynote speaker, Neuroscience Graduate Program, Annual Student/Faculty Retreat, University of Cincinnati, Cincinnati, OH, March 26, 2004)

"Growth Factors, Gene Therapy, and Parkinson's Disease"
(Symposium speaker, Central Ohio Parkinson Society, Annual Symposium, Columbus, OH, April 24, 2004)

"Neuroprotective and Neurotrophic Actions of Neuregulin Polypeptides in the Dopaminergic Nigrostriatal System" (Symposium speaker; Summer Neuropeptide Conference, Miami Beach, FL, July 7, 2004)

"Neuregulins and CNS Neurodegenerative Disorders: An Update"
(Grand Rounds, Dept. of Neurology, University of Cincinnati, Cincinnati, OH, March 16, 2005)

"Neuregulins: Novel Trophic Factors for Dopaminergic Neurons"
(Symposium speaker, Ohio Miami Valley Chapter, Society for Neuroscience, Annual Symposium/ Business Meeting, Miami University, Oxford, OH, January 4, 2006)

"Neuregulins as Novel Trophic Factors for Midbrain Dopaminergic Neurons: Implications for Parkinson's Disease" (Invited Seminar, College of Pharmacy, University of Cincinnati, Cincinnati, OH, December 6, 2006)

List of personnel receiving pay from the research effort:

Kim B. Seroogy, Ph.D. (UK and UC)
David M. Yurek, Ph.D. (UK)
Lixin Zhang (graduate student; UK)
Kerstin Lundgren (technician: UK)
Jon Dickerson (graduate student; UC)
Ann Hemmerele (graduate student; UC)

Degree Obtained Partially Supported by this Award:

Ph.D., Dissertation, Lixin Zhang, University of Kentucky (2004)

Funding applied for (and received) based on work supported by this award:

Agency: Michael J. Fox Foundation for Parkinson's Research
Title: Neuregulin-2β: A Novel Trophic Factor for Midbrain Dopamine Neurons
Role: PI: Kim B. Seroogy
Period: 01/01/05 – 09/30/07

CONCLUSIONS

Results from studies supported by the present grant provide good evidence that neuregulins can function as neurotrophic and neuroprotective factors for nigrostriatal dopaminergic neurons. Studies in vivo demonstrated that administration of neuregulins to the normal nigrostriatal system enhances striatal dopamine release. This is important because compounds that can stimulate the secretion or release of dopamine in the nigrostriatal system have the potential for overcoming the lack of dopamine neuronal function in Parkinson's disease patients. Results from in vitro experiments show that the neuregulin GGF2 protects cultured midbrain neurons against injury, most notably neurotoxin-induced degeneration. In addition, treatment of the cultures with GGF2 promotes the survival, neurite outgrowth and dopamine uptake of the developing dopamine cells. Thus, GGF2 exhibits neurotrophic and neuroprotective actions for mesencephalic dopamine neurons. Finally, our additional in vivo studies show that infusion of GGF2 significantly protects nigral dopamine neurons against neurotoxin-induced degeneration and significantly improves behavioral functional recovery. Taken together, data from these studies indicate that neuregulins are dopamine-specific neurotrophic factors that may have therapeutic potential for the treatment of Parkinson's disease.

APPENDICES

I. Figures 1-7 from Body of Report

II. Publications (reprints; pdf files)

Zhang, L., Fletcher-Turner, A., Lundgren, K.H., Marchionni, M., Yurek, D.M. and Seroogy, K.B. (2004) Neurotrophic and neuroprotective effects of the neuregulin glial growth factor-2 on dopaminergic neurons in rat primary midbrain cultures. *Journal of Neurochemistry* 91: 1358-1368.

Yurek, D.M., Zhang, L., Fletcher-Turner, A. and Seroogy, K.B. (2004) Supranigral injection of neuregulin1-β induces striatal dopamine overflow. *Brain Research* 1028: 116-119.

Seroogy, K.B. and Zhang, L. (2006) Neuregulins. In: Handbook of Biologically Active Peptides, A.J. Kastin, (ed.), Elsevier Academic Press, Amsterdam, pp. 1401-1407.
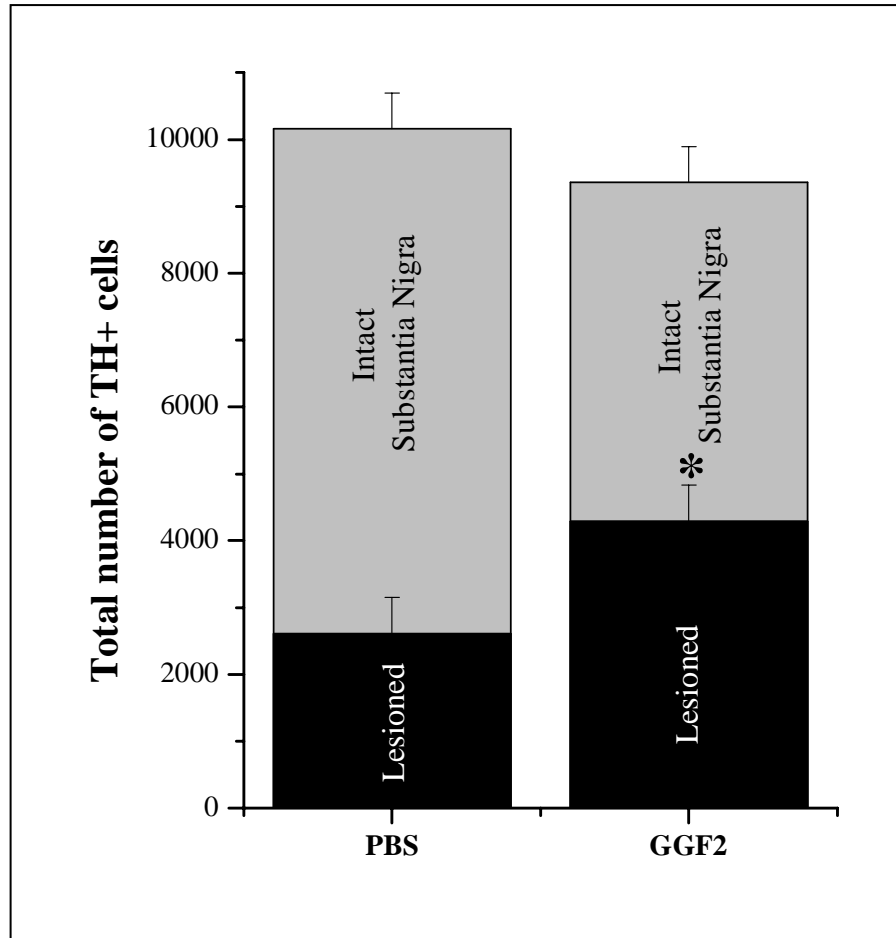
APPENDIX I

Fig. 1

Fig. 1. Supranigral infusion of GGF2 protects midbrain dopaminergic neurons against neurotoxin-induced degeneration. Immunostaining for TH in rat midbrain sections. At 5 weeks post-lesion, the rats were perfused with 4% paraformaldehyde and immunocytochemistry was performed to detect the dopaminergic neurons (TH+) in the ventral mesencephalon. More TH+ cells are observed in the substantia nigra on the side of the lesion (right) in the animal receiving GGF2 treatment when compared to PBS treatment. Arrows indicate cannula tracts.

Fig. 2

Fig. 2. Supranigral infusion of GGF2 protects midbrain dopaminergic neurons against neurotoxin-induced degeneration: Quantification. The number of TH+ neurons within the substantia nigra (pars compacta and pars lateralis) was counted bilaterally by unbiased stereological analysis using the optical fractionator method. Every sixth 30-μm-thick coronal tissue section through the substantia nigra was sampled for evaluation using a Bioquant image analysis system. This analysis revealed significantly more TH+ cells present in the lesioned nigra of the GGF2 treatment group when compared to the PBS control group. *p<0.05 vs. PBS.

Fig 3.

Cylinder test score

Fig. 3. Supranigral infusion of GGF2 promotes functional recovery after neurotoxin lesions of the nigrostriatal system. Forelimb asymmetry (cylinder) tests were recorded at 2 and 4 weeks post-lesion. Scores were analyzed using two-way ANOVA and presented as the percent limb usage for the impaired limb only. Compared to PBS-treated animals, GGF2-treated animals showed a significantly greater usage of the impaired forelimb at 4 weeks post-lesion. The dotted line indicates level of normal limb function. *p< 0.05 vs. PBS at 4 weeks.
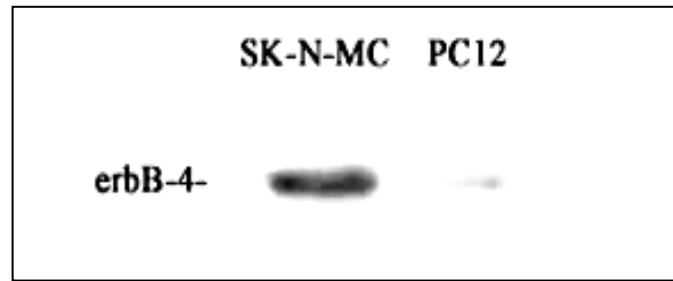
Fig. 4.

Fig. 4. Western blot analysis of ErbB4 receptor protein in human SK-N-MC cells. ErbB4 (appropriate size band at 185 kD) is readily detected in the SK-N-MC cells, but is absent in PC12 cells, which normally do not express the neuregulin receptor.
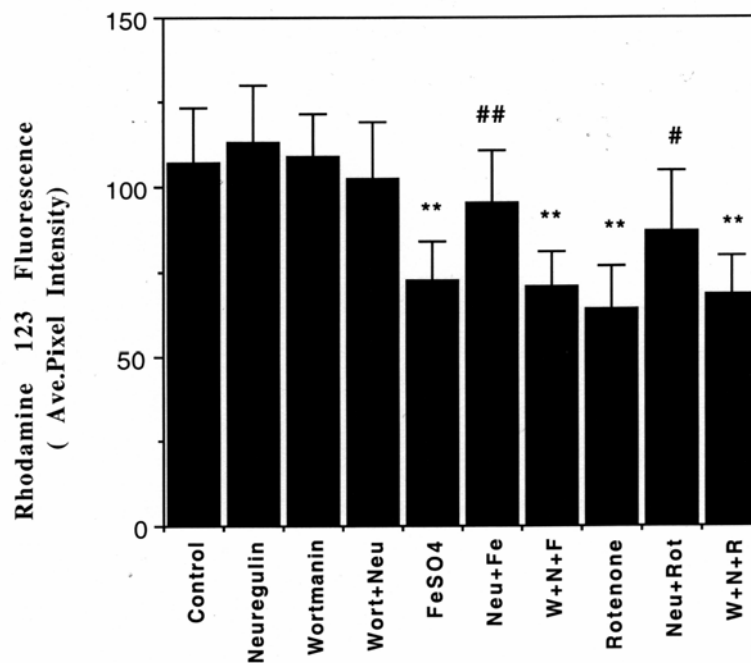
Fig. 5.

Fig. 5. Inhibition of PI3-kinase attenuates the protective effect of neuregulin against mitochondrial dysfunction in SK-N-MC cells. Cells were pretreated for 24 h with 100 ng/ml of the neuregulin HRG-β1 followed by oxidative (FeSO$_4$, 10 μM) and metabolic (rotenone, 1 μM) injury and a 24h survival period. Note that neuregulin pretreatment (column 6) significantly protected the cells against against the iron-induced decrease in mitochondrial membrane integrity (column 5). Pretreatment with the PI3-kinase inhibitor wortmannin completely abrogated this protective effect (column 7). Similar results were obtained for the rotenone experiments (columns 8-10). **p = 0.05 vs control; #p = 0.05 vs rotenone and W+N+R); ##p = 0.05 vs FeSO$_4$ and W+N+Fe).
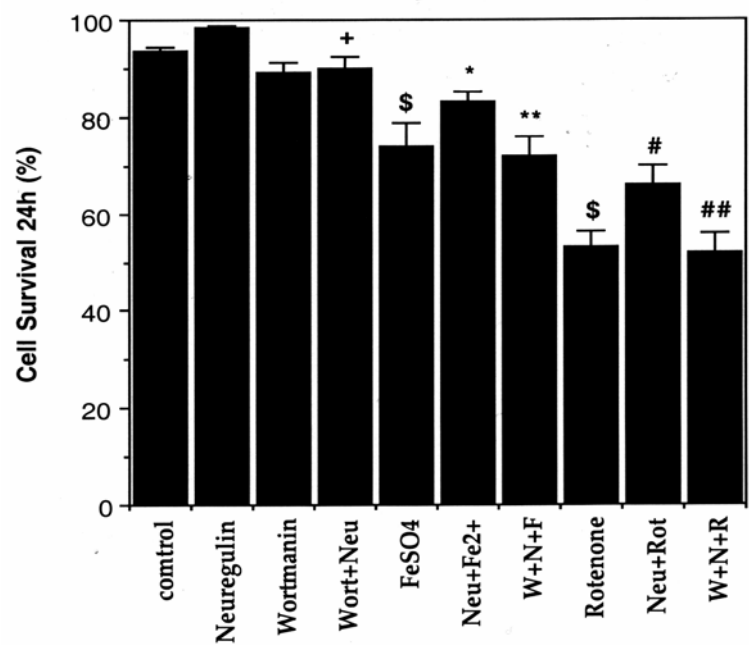
Fig. 6.

Fig. 6. Neuroprotective effects of neuregulin on survival of dopaminergic SK-N-MC cells after oxidative and metabolic insults: prevention by inhibition of PI3-kinase. Cells were pretreated for 24 h with 100 ng/ml of the neuregulin HRG-β1 followed by oxidative (FeSO$_4$, 10 μM) and metabolic (rotenone, 1 μM) injury and a 24h survival period. Note that neuregulin pretreatment (column 6) significantly protected the cells against against the iron-induced decrease in cell survival (column 5). Pretreatment with the PI3-kinase inhibitor wortmannin completely abrogated this protective effect (column 7). Similar results were obtained for the rotenone experiments (columns 8-10).
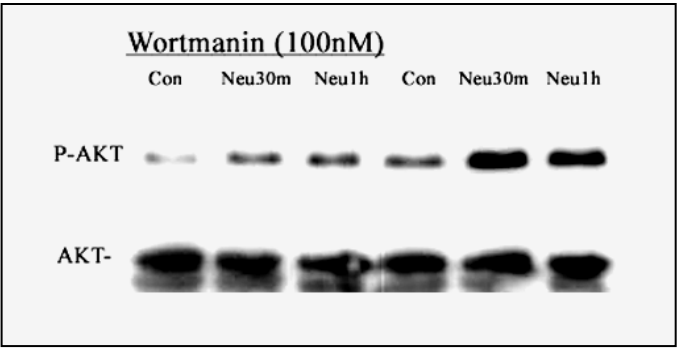
Fig. 7.

Fig. 7. The PI3-kinase/AKT pathway is a potential mediator of neuregulin neuroprotection in SK-N-MC cells. Addition of neuregulin to the cultures for 30 min or 1h increased levels of phosphorylated-AKT (but not total AKT) compared to controls (right 3 columns). Pretreatment of the cells with the PI3-kinase inhibitor wortmannin attenuated this neuregulin-induced increase (left 3 columns).

Neurotrophic and neuroprotective effects of the neuregulin glial growth factor-2 on dopaminergic neurons in rat primary midbrain cultures

Lixin Zhang,* Anita Fletcher-Turner,† Mark A. Marchionni,‡ Subbu Apparsundaram,*
Kerstin H. Lundgren,§ David M. Yurek† and Kim B. Seroogy*,§

Department of Anatomy & Neurobiology, University of Kentucky, Lexington, Kentucky, USA

†*Department of Surgery/Neurosurgery, University of Kentucky, Lexington, Kentucky, USA*

‡*CeNeS Pharmaceuticals Inc., Norwood, Massachusetts, USA*

§*Department of Neurology, University of Cincinnati, Cincinnati, Ohio, USA*

Abstract

Glial growth factor-2 (GGF2) and other neuregulin (NRG) isoforms have been shown to play important roles in survival, migration, and differentiation of certain neural and non-neural cells. Because midbrain dopamine (DA) cells express the NRG receptor, ErbB4, the present study examined the potential neurotrophic and/or neuroprotective effects of GGF2 on cultured primary dopaminergic neurons. Embryonic day 14 rat mesencephalic cell cultures were maintained in serum-free medium and treated with GGF2 or vehicle. The number of tyrosine hydroxylase-positive (TH+) neurons and high-affinity [³H]DA uptake were assessed at day *in vitro* (DIV) 9. Separate midbrain cultures were treated with 100 ng/mL GGF2 on DIV 0 and exposed to the catecholamine-specific neurotoxin 6-hydroxydopamine (6-OHDA) on DIV 4. GGF2 treatment

significantly increased DA uptake, the number of TH+ neurons, and neurite outgrowth when compared to the controls in both the serum-free and the 6-OHDA-challenged cultures. Furthermore, three NRG receptors were detected in the midbrain cultures by western blot analysis. Immunostaining for glial fibrillary acidic protein revealed that GGF2 also weakly promoted mesencephalic glial proliferation in the midbrain cultures. These results indicate that GGF2 is neurotrophic and neuroprotective for developing dopaminergic neurons and suggest a role for NRGs in repair of the damaged nigrostriatal system that occurs in Parkinson's disease.

Keywords: dopamine, glial growth factor, glial growth factor-2, neuroprotection, neurotrophic factor, Parkinson's disease.
J. Neurochem. (2004) **91,** 1358–1368.

Parkinson's disease (PD) is a major neurodegenerative disorder involving the progressive degeneration of dopaminergic neurons in the nigrostriatal pathway (Grunblatt *et al.* 2000; Obeso *et al.* 2000; Blum *et al.* 2001). The loss of dopaminergic neurons in the substantia nigra results in a depletion of dopamine (DA) within the target nuclei of the basal ganglia, particularly the putamen. One promising therapeutic approach for treatment of the disease is the use of neurotrophic factors to promote the survival and growth of dopaminergic neurons. The ultimate goal is to slow or halt neuronal degeneration at an early stage in order to preserve existing DA neurons and possibly to stimulate compensatory growth in these same cells (Lindsay *et al.* 1993; Moller *et al.* 1996; Collier and Sortwell 1999).

Neuregulins (NRGs) are a family of structurally related polypeptide growth and differentiation factors produced by

four genes termed NRG 1–4. They have been shown to play roles in cell survival, migration, and differentiation in both neural and non-neural cells (reviewed in Burden and Yarden 1997; Gassmann and Lemke 1997; Yarden and Sliwkowski 2001; Falls 2003). NRGs and their ErbB family of protein

tyrosine kinase receptors are broadly expressed in multiple regions of developing and adult rat brains, and both neuronal and glial cells have been identified as NRG-producing cells (Meyer and Birchmeier 1994; Pinkas-Kramarski *et al*. 1994; Bermingham-McDonogh *et al*. 1996, 1997; Steiner *et al*. 1999). The most well-known NRG-associated functions within the nervous system include roles in glial cell proliferation, maturation and survival, neuromuscular acetylcholine receptor induction, and early neuronal migration and survival (Dong *et al*. 1995; Canoll *et al*. 1996; Rio *et al*. 1997; Raabe *et al*. 1998; Marchionni *et al*. 1999; Adlkofer and Lai 2000). NRGs have also been found to regulate the proliferation of neuroepithelia and the differentiation of retinal ganglion cells (Zhao and Lemke 1998), and are modulated in response to traumatic brain injury and stroke (Tokita *et al*. 2001; Parker *et al*. 2002). In addition to playing essential roles in nervous, heart and other organ system development, NRGs are also implicated in the pathogenesis of several human diseases, including schizophrenia, multiple sclerosis and breast cancer (Krane and Leder 1996; Cannella *et al*. 1999; Stefansson *et al*. 2002). *In vitro* studies have shown that NRG stimulates neurite outgrowth in PC12 cells and cerebellar granule cells, and rescues PC12-ErbB4 cells from apoptotic cell death induced by H_2O_2 (Rieff *et al*. 1999; Villegas *et al*. 2000; Goldshmit *et al*. 2001). However, despite the expanding roles of NRG in the CNS described above, little is known of the functions of NRGs in the developing and mature midbrain.

Recent studies have demonstrated that the functional NRG receptor, ErbB4, is expressed within a substantial population of cells within the substantia nigra and ventral tegmental area of rodents (Steiner *et al*. 1999; Gerecke *et al*. 2001; Yurek and Seroogy 2001). These findings raise the possibility that ventral mesencephalic DA neurons may be responsive to the trophic effects of NRGs. In the present study, we investigated the neurotrophic effects of NRG on dopaminergic neurons using primary rat mesencephalic cell cultures. Various doses of glial growth factor-2 (GGF2), a potent and well-characterized isoform of the NRG-1 gene, were tested for neurotrophic/neuroprotective actions in the midbrain cultures under both serum-free and neurotoxic conditions. The identity of NRG receptor subtypes, as well as the effects of GGF-2 treatment upon glial cell proliferation, within the midbrain cultures was also analyzed.

Materials and methods

Culture medium and chemicals
Serum-free medium consisted of equal volumes of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture supplemented with $1 \times N2$ supplement, 2.5 μg/mL fungizone, and 2 μL/mL Gentamicin (all from Gibco, Grand Island, NY, USA). Fetal bovine serum, fetal calf serum, and trypsin were also obtained from Gibco. DNase was purchased from Worthington (Biochemical Corporation, Lakewood, NJ, USA). All other chemicals used were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated. Antibodies used in this study: primary antisera to tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP) and neuron-specific enolase (NSE) were purchased from Chemicon (Temecula, CA, USA); antisera to ErbB2, ErbB3 and ErbB4 were from NeoMarkers (Labvision Corporation, Fremont, CA, USA); all the secondary antibodies were from Chemicon.

Mesencephalic cell cultures
Timed-pregnant Sprague Dawley rats were purchased from Harlan (Indianapolis, IN, USA) and midbrain cell cultures were established from mesencephalic tissue dissected from embryonic day 14 (E14) fetuses using the protocols described by Takeshima *et al*. (1996) and Sortwell *et al*. (2000), with minor modifications. This procedure generates a highly neuron-enriched culture with 10–15% DA cells at the time of plating. Briefly, using sterile techniques, ventral mesencephalic tissue was dissected from rat embryos and pooled in an oxygenated, cold, sterile calcium- and magnesium-free buffer. Cell suspensions of embryonic mesencephalic tissue were prepared through a series of calcium- and magnesium-free buffer rinses, incubated in 0.125% trypsin, rinsed in calcium- and magnesium-free buffer again, and triturated in 0.004% DNase to disperse the cells into solution. The cells were pelleted at 164 *g* for 10 min and then re-suspended in 1 mL of growth medium. Trypan blue was added to a sample of the cell suspension and viewed in a hemocytometer to determine cell viability and cell numbers. Twenty-four-well culture plates were pre-coated with poly–D-lysine (5 mg/50 mL) and cells were plated at a concentration of 4000 cells/μL and 25 μL per well using the microisland method (Takeshima *et al*. 1996). Two hours after plating, 1 mL of Dulbecco's modified Eagle's medium/F12 medium was added to each well. In separate cultures, for the 6-hydroxydopamine (6-OHDA) study, Dulbecco's modified Eagle's medium/F12 with 5% fetal bovine serum was added to each well. The entire medium was changed after the first 24 h and every second day thereafter. All cell cultures were kept in a water-jacketed tissue culture incubator at 37°C with 5% CO_2/95% air mixture.

Treatments of cultures
Recombinant human glial growth factor-2 (rhGGF2; provided by CeNeS Pharmaceuticals Inc.) was diluted to a stock concentration of 100 μg/mL in distilled water. At day *in vitro* (DIV) 0, GGF2 (10, 50, or 100 ng/mL) was added to each treatment well and maintained throughout the experiments. The same amount of distilled water without the growth factor was added to the medium in control wells. In the experiments involving 6-OHDA challenge, GGF2 (100 ng) was added to treatment wells at DIV 0. At DIV 4, some cells were exposed to freshly prepared 6-OHDA (50 μM) for 45 min at 37°C. At the end of the 6-OHDA treatment, cultures were washed with Dulbecco's modified Eagle's medium three times and re-fed with fresh Dulbecco's modified Eagle's medium/F12 medium, and then GGF2 treatment was resumed until the final day of culture.

In situ hybridization
Fresh-frozen, cryostat-cut, slide-mounted sections through the E14 rat mesencephalon were processed for the detection of ErbB4 and TH mRNAs by using *in situ* hybridization with ^{35}S-labeled cRNA

probes, as described previously (Numan and Seroogy 1997; Kornblum *et al.* 2000; Zhang *et al.* 2000). The cDNA template used to produce the ErbB4 cRNA was kindly supplied by H. Kornblum (UCLA; Kornblum *et al.* 2000). After post-hybridization ribonuclease treatment and washes, the sections were exposed to β-Max Hyperfilm (Amersham, Little Chalfont Buckinghamshire, UK) for 3–6 days to generate film autoradiograms, and then coated with NTB2 nuclear track emulsion (Kodak, Rochester, NY; 1 : 1 in H_2O) for 3–4 weeks to generate emulsion autoradiograms.

Immunocytochemistry

Culture medium was removed and the cells were rinsed in Tris buffer (pH = 7.3), fixed in 4% paraformaldehyde for 20 min, and rinsed again in Tris. Non-specific staining was blocked with 10% goat serum for 1 h. Cells were incubated in primary antisera to TH (1 : 4000), GFAP (1 : 800), or NSE (1 : 200) overnight at 4°C. After several Tris rinses, cells were incubated in the following biotin-conjugated secondary antibodies for 1 h: goat anti-mouse IgG (1 : 800) for TH or goat anti-rabbit IgG (1 : 400) for GFAP and NSE. Sections were taken through a series of rinses and then incubated in ABC-peroxidase reagent (Vector, Burlingame, CA, USA) for 1 h. Following rinses, antisera labeling was visualized by exposure to 0.5 mg/mL 3,3′-diaminobenzidine and 0.03% H_2O_2 in Tris buffer.

High-affinity dopamine uptake

The DA uptake assay followed the protocol described by Kramer *et al.* (1999), with some modifications (Apparsundaram *et al.* 1998). Cultures were rinsed with Krebs–Ringer's–Henseleit buffer (125 mM NaCl, 5 mM KCl, 1.5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.5 mM KH_2PO_4, 10 mM D-glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline, and 0.1 mM ascorbic acid and saturated with 95% O_2/5% CO_2, pH 7.4) and incubated with the same buffer containing [^{3}H]DA (0.5 μCi/mL; specific activity 40 Ci/mmol; NEN Life Sciences Products Inc, Boston, MA, USA) for 10 min at 37°C. The cultures were then rinsed twice and the accumulated [^{3}H]DA was released by incubation with 0.5 mL 95% ethanol. The solution was added to 5 mL Ecoscint A and counted in a scintillation spectrophotometer. Nonspecific labeling of [^{3}H]DA was measured by adding 10 μM nomifensine to the incubation medium.

Western blots

Western blots were performed on dissociated mesencephalic cultures. Cells were gently rinsed with cold phosphate-buffered saline, harvested using a cell scraper, and lysed in buffer containing 25 mM Hepes, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and 10 mM dithiothreitol on ice for 30 min. Lysates were centrifuged at 160 000 *g* at 4°C for 20 min, and the supernatant was stored at −80°C. Protein concentration was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The protein was denatured by boiling at 100°C for 10 min. An aliquot (50 μg as protein) of the supernatant with 10 μL loading buffer (Bio-Rad, Hercules, CA, USA) was loaded on a sodium dodecyl sulfate–polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked in 5% skim milk in Tris-buffered saline plus Tween (150 mM NaCl, 10 mM Tris-HCl, 0.05% Tween-20; pH 8.8) for 1 h at room temperature, then incubated with primary antibody [anti-ErbB2 (1 : 200), anti-ErbB3 (1 : 200), anti-ErbB4 (1 : 200), anti-GFAP (1 : 1000)] overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 10 000). Subsequently, membrane-bound horseradish peroxidase-labeled antibodies were detected by an enhanced chemiluminescence detection system (ECL-Plus; Amersham) and exposed to Hyperfilm ECL (Amersham).

Cell counts and morphological analyses

The number of dopaminergic neurons or astroglial cells in cultures was determined by counting the cells immunostained for TH or GFAP. The cells were counted manually by an observer, blinded to treatment condition, in nine adjacent fields located in the center of the microisland at a 20 × magnification; this area accounts for approximately 25% of the island and approximately 40% of the cell population (Sortwell *et al.* 2000). For analysis of neurite morphology, 90 TH+ neurons from each treatment group were randomly selected and the length of the longest neurite of each neuron was measured by its intersection with a grid (the length of one grid unit is 50 μm), and the number of fibers per TH+ cell was also counted under 40 × magnification.

Statistical analysis

All experiments were performed at least in triplicate with a minimum sample size per run of three wells per group. Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test. The computer program SigmaStat Version 2.0 was used for statistical analysis.

Results

Cell viability for all cultures was 95% or greater and approximately 10–15% of the total number of plated cells were TH+ at 12 h after plating. Under serum-free conditions, GFAP+ cells were not observed until DIV 9.

Glial growth factor-2 promotes dopamine cell survival under serum-free conditions

Cultures maintained in serum-free Dulbecco's modified Eagle's medium/F12 medium were fixed with 4% paraformaldehyde and immunostained for TH at DIV 1, 4 or 9. In parallel cultures, high-affinity [^{3}H]DA uptake was performed at DIV 9. Figure 1 illustrates the time course of the effect of GGF2 on the number of surviving TH+ cells. Under serum-free and growth factor-free conditions, the number of DA cells decreased dramatically from DIV 4 (∼25 TH+ cells/field) to DIV 9 (∼3 TH+ cells/field). Although the number of TH+ cells declined from DIV 1 until DIV 9 in the GGF2-treated cells, the number of TH+ cells at DIV 9 for this group was significantly greater (threefold higher) than counts for TH+ cells in control cultures at DIV 9. This effect was dose-dependent; as Fig. 2 shows, the number of TH+ cells increased proportionally with the dose of GGF2. Furthermore, high-affinity DA uptake confirmed the immunocytochemical results. Compared to control cultures, the value of DA uptake was increased 1.5-fold by GGF2 treatment. These
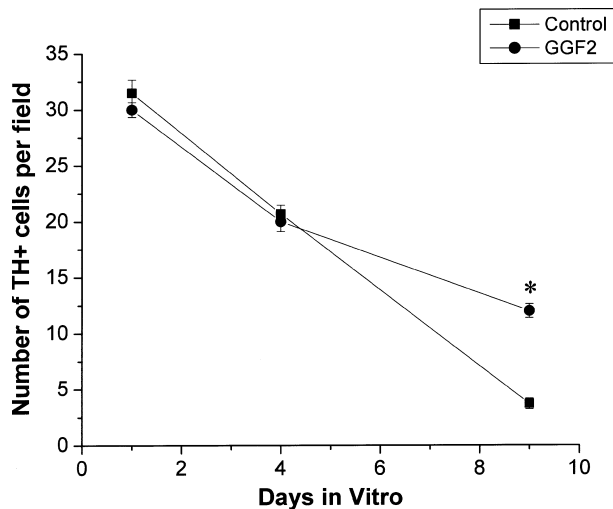
Fig. 1 Cultures maintained in serum-free medium were treated with either 100 ng glial growth factor-2 (GGF2) or vehicle and fixed with 4% paraformaldehyde on days *in vitro* (DIV) 1, 4 and 9. Tyrosine hydroxylase-positive (TH+) cells were counted at the three time points. GGF2 significantly attenuated TH+ cell death on DIV 9 in comparison with control cultures; *$p < 0.05$ vs. control.
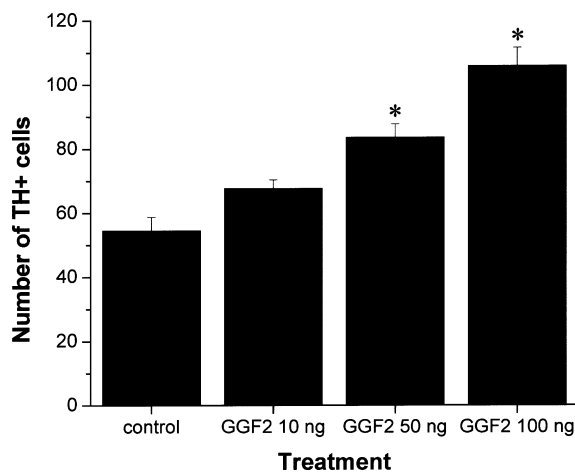

Fig. 3 Parallel midbrain cultures to those shown in Figs 1 and 2 were used for this study. On day *in vitro* (DIV) 9, cultures were analyzed for dopamine uptake. As the figure shows, 100 ng glial growth factor-2 (GGF2) treatment significantly increases dopamine uptake when compared to control cultures; *$p < 0.05$ vs. control.

Fig. 2 Midbrain cultures maintained in serum-free medium were treated with various doses of glial growth factor-2 (GGF2) or vehicle (control). On day *in vitro* (DIV) 9, the cells were fixed and tyrosine hydroxylase (TH) immunostaining was performed. The number of surviving TH+ cells in the cultures was increased in proportion to the dose of GGF2; *$p < 0.05$ vs. control.

Fig. 4 Tyrosine hydroxylase (TH) immunostaining of midbrain cultures at day *in vitro* (DIV) 9. Serum-free medium midbrain cultures were treated with vehicle (a), 10 ng glial growth factor-2 (GGF2) (b), 50 ng GGF2 (c), or 100 ng GGF2 (d) at DIV 0. Compared to the control cultures, TH+ cells in GGF2-treated cultures exhibited longer and more numerous neurites. This effect is more obvious at the higher doses of GGF2. Scale bar = 50 μm.

data are consistent with the TH+ cell counts and also show a dose-dependent relationship with GGF2 treatment (Fig. 3).

Glial growth factor-2 enhances neuritic outgrowth of dopaminergic neurons

We observed that the DA cells in GGF2-treated cultures not only were present at a higher density, but that neuritic
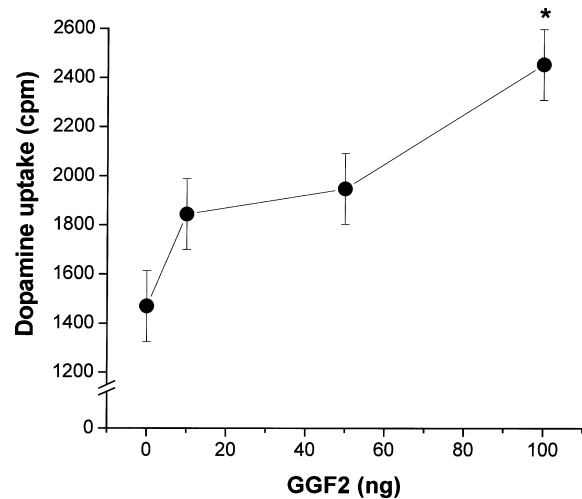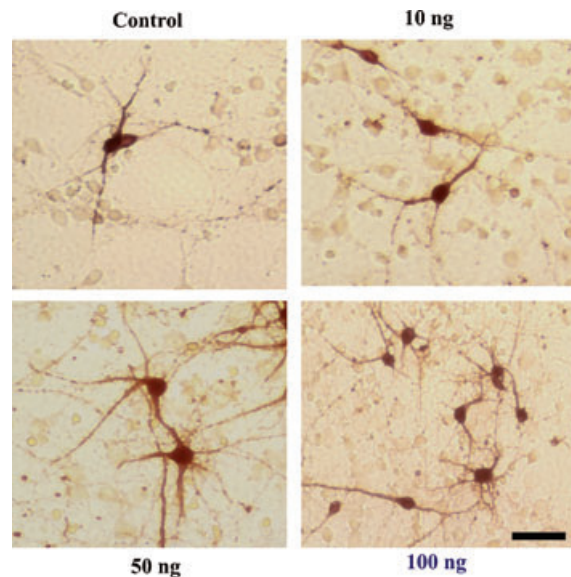
outgrowth appeared more robust than in control cultures. Figure 4 illustrates morphological differences of TH+ cells in each treatment group. Dopamine cells treated with 50 or 100 ng GGF2 exhibited robust neuritic outgrowth, had more

fiber branches, and extended longer neurites when compared to control or 10 ng GGF2-treated cultures. These morphological observations were confirmed by measurements of the longest neurite and number of fibers per TH+ cell from randomly selected TH+ cells in each treatment group at DIV 9 (Figs 5a and b). The average length of the longest neurite per TH+ cell was 146.5 μm in 100 ng GGF2-treated cultures, but only 60.1 μm in control cultures. The majority of TH+ cells (90%) treated with GGF2 had 2–5 neuritic branches, whereas 80% of TH+ cells in control cultures had only 1–2 neuritic branches ($p < 0.001$; $n = 90$). Thus, these data suggest TH+ cells treated with GGF2 have longer neuritic processes and more branches. This trophic effect of GGF2 on DA cells occurred at the lowest dose of GGF2 tested (10 ng) and became more pronounced at the higher doses.
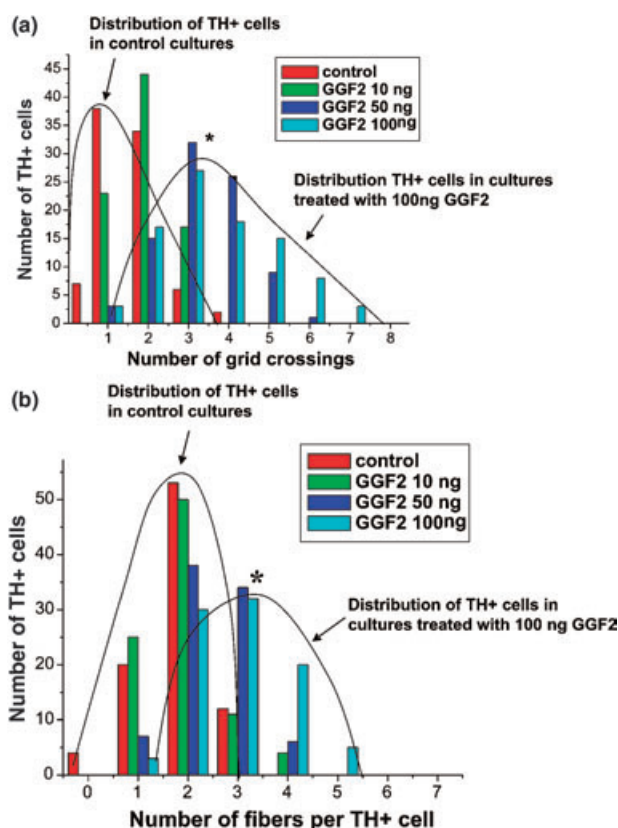

Fig. 5 (a) The longest fiber of each tyrosine hydroxylase-positive (TH+) cell was measured at day *in vitro* (DIV) 9 in serum-free midbrain cell cultures. Most untreated TH+ cells extended fibers that crossed 1.0–2.5 grid units. In contrast, in glial growth factor-2 (GGF2)-treated cultures, most TH+ cells extended fibers that crossed 2.5–6 grid units. One grid unit = 50 μm. (b) The number of fibers per TH+ cells in control vs. GGF2-treated cultures. The majority of untreated TH+ cells exhibited 0–2.5 fibers, whereas the GGF2 (50, 100 ng)-treated TH+ cells extended 1.5–5.5 fibers. *$p < 0.05$ for 100 ng GGF2 vs. control.

Glial growth factor-2 protects dopaminergic neurons from 6-hydroxydopamine-induced cell death

The possible neuroprotective effect of GGF2 on dopaminergic cells also was examined in this study. Midbrain cell cultures prepared from E14 rats as described above were maintained in Dulbecco's modified Eagle's medium/N2 medium supplemented with 5% fetal bovine serum during the first 24 h, and then switched to serum-free Dulbecco's modified Eagle's medium/N2 medium. The cultures were exposed to 50 μM 6-OHDA for 45 min on DIV 4 and DA uptake or immunohistochemical analysis was performed after 48 h and 72 h, respectively. As Fig. 6 shows qualitatively, TH+ cells treated with GGF2 exhibited enhanced neuritic outgrowth with a 'healthier' appearance, compared to the cultures treated with 6-OHDA only. Both the number of surviving TH+ cells and the value of DA uptake were significantly increased by GGF2 treatment in comparison with control cultures exposed to 6-OHDA (Figs 7a and b). The number of TH+ cells was reduced to 50% of control levels after 6-OHDA treatment; however, 100 ng GGF2 treatment prevented this cell death and maintained TH+ cell numbers at control levels (Fig. 7a). Similarly, DA-uptake analysis also showed that whereas a 50% loss was observed in cultures treated with 6-OHDA only (Fig. 7b), GGF2 treatment in the neurotoxin-challenged cultures attenuated the reduction in DA uptake to only 20% of control values, a statistically significant effect. This suggests that GGF2 protected a significant portion of the DA neurons from 6-OHDA-induced degeneration.

Neuregulin receptor expression

To determine if the dissociated primary midbrain cell cultures expressed NRG receptors, western blots for ErbB2, ErbB3, and ErbB4 were performed on the midbrain cultures at DIV 1, 4, and 9. As Fig. 8 shows, all three of the NRG receptors were expressed in midbrain cultures as early as DIV 1. This expression continued until DIV 9 in both control and GGF2-treated cultures. Quantitative analyses of band densities for each receptor did not reveal statistically significant differences in receptor protein levels among the time points examined nor between control and GGF2-treated groups (data not shown).

The primary midbrain cultures for all of these *in vitro* experiments are derived from the E14 rat ventral mesencephalon. To verify that the ErbB4 NRG receptor is present in cells of this region at the time of tissue harvesting, we performed *in situ* hybridization for TH and ErbB4 mRNAs on adjacent sagittal sections through the brain of the rat fetus (Fig. 9). Intense labeling for TH (Fig. 9b) and ErbB4 (Fig. 9c) was observed in the region of the ventral mesencephalic flexure in adjacent sections. Note that ErbB4 mRNA was also widely distributed throughout other embryonic brain regions. When merging the adjacent sections, labeling for TH and ErbB4 mRNAs directly overlapped in the ventral
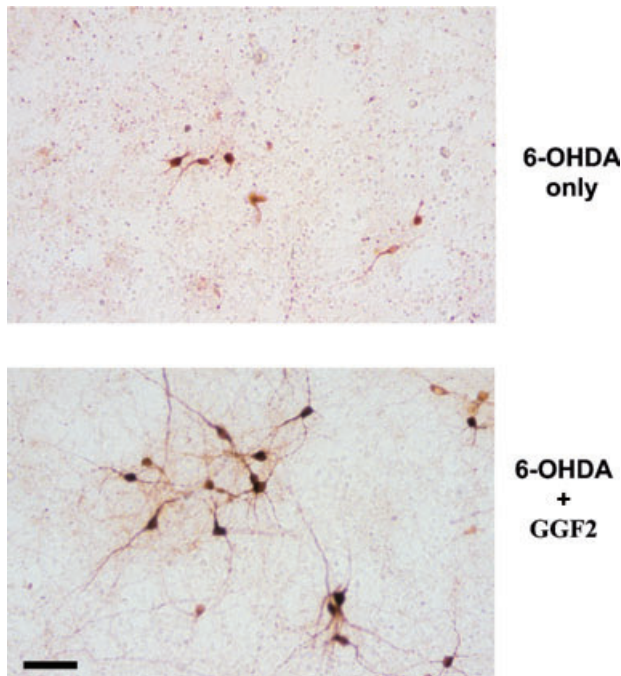
Fig. 6 Glial growth factor-2 (GGF2) protects tyrosine hydroxylase-positive (TH+) neurons from 6-hydroxydopamine (6-OHDA)-induced degeneration. Immunostaining for TH in midbrain cultures challenged with 6-OHDA alone (top panel) or treated with GGF2 (100 ng) and then challenged with 6-OHDA (bottom panel). Scale bar = 100 μm.

mesencephalic region (Fig. 9d), indicating that tissue dissected from this region at this time would contain numerous ErbB4-expressing cells.

Effect of glial growth factor-2 on mesencephalic glia

To initially test if the trophic effects of GGF2 on developing dopaminergic neurons may indirectly involve mesencephalic glia, immunocytochemical and western blot analyses for GFAP were performed in parallel midbrain cultures. Serum-free midbrain cultures were treated with various doses of GGF2 or vehicle. The cells were either fixed with 4% paraformaldehyde for GFAP immunostaining or harvested for western blots on DIV 1, 4 and 9. GFAP+ cells were not detected at DIV 1 or 4 in either control or GGF2-treated cultures. However, at DIV 9 a few GFAP+ cells were present in cultures treated with either 50 ng (2–3 per field) or 100 ng (4–5 per field) GGF2 (Fig. 10). The number of GFAP+ cells showed a significant increase in cultures treated with 50 and 100 ng/mL GGF2 (Fig. 10), although the total number of GFAP+ cells was still extremely low relative to the total population of cells at this stage of the culture. Consonant with the immunocytochemical data, no GFAP protein was detected until DIV 9 in the cultures treated with 50 and 100 ng/mL GGF2, as determined by western blot analysis. Quantification of the band densities in DIV 9 cultures
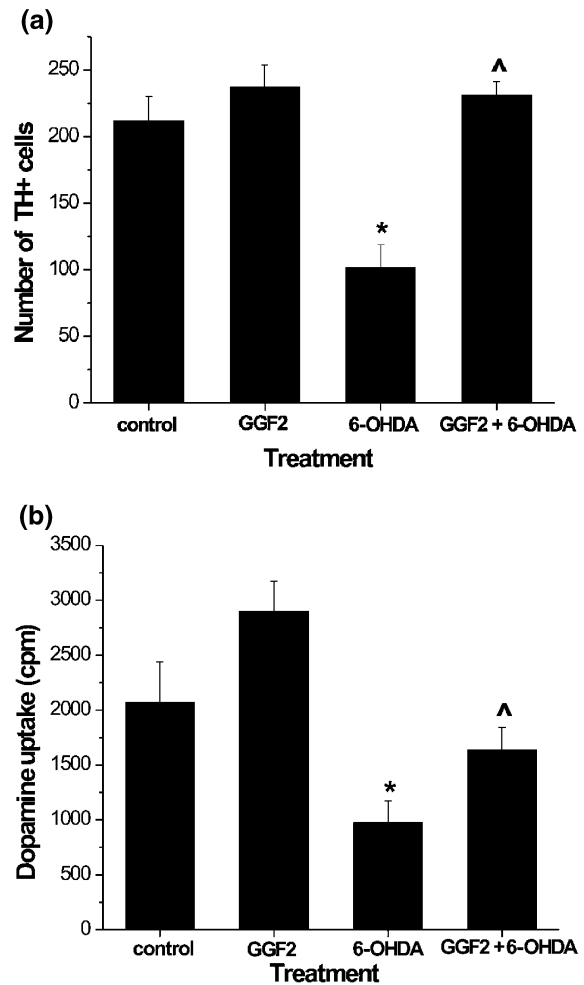

Fig. 7 (a) The number of tyrosine hydroxylase-positive (TH+) cells in midbrain cell cultures challenged with 6-hydroxydopamine (6-OHDA). Note that 6-OHDA treatment induced about a 50% loss of TH+ cells compared to the control level; this cell loss was prevented by treatment with 100 ng glial growth factor-2 (GGF2). (b) Dopamine uptake was performed on midbrain cell cultures challenged with 6-OHDA. 6-OHDA treatment reduced the level of dopamine uptake to 50% of the control value. Treatment with 100 ng GGF2 restored the level to 80% of the control value; *$p < 0.05$ vs. control, ^$p < 0.05$ vs. 6-OHDA only.
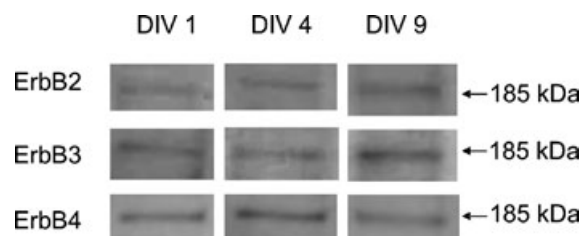

Fig. 8 Western blots for neuregulin receptors. Western blots for ErbB2, ErbB3, and ErbB4 were performed on serum-free medium midbrain cell cultures at days *in vitro* (DIV) 1, 4 and 9. All three receptors were detected at each of the three time points.

E14 Fetus / **E14 TH mRNA**
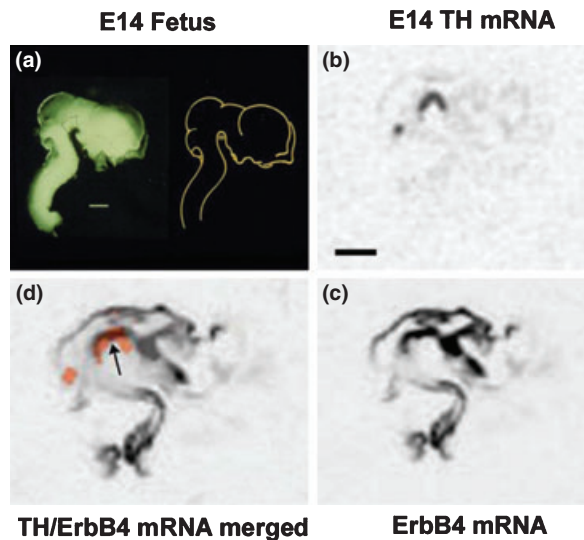**TH/ErbB4 mRNA merged** / **ErbB4 mRNA**

Fig. 9 *In situ* hybridization for tyrosine hydroxylase (TH) and ErbB4 mRNAs in E14 rat fetus. The left side of (a) shows a sagittal view of an E14 rat fetus CNS and the right side is a sagittal diagram of the E14 rat CNS. Two adjacent sections (b, c) taken from an E14 fetus show robust expression of TH (b) and ErbB4 (c) mRNAs in the ventral mesencephalic flexure region. Notice there is a substantial overlap in the expression of both mRNAs (TH mRNA labeling indicated in orange, ErbB4 mRNA labeling in black) in the mesencephalic flexure (arrow) when these two sections are merged into one image (d). Scale bar in (a) = 1 mm.
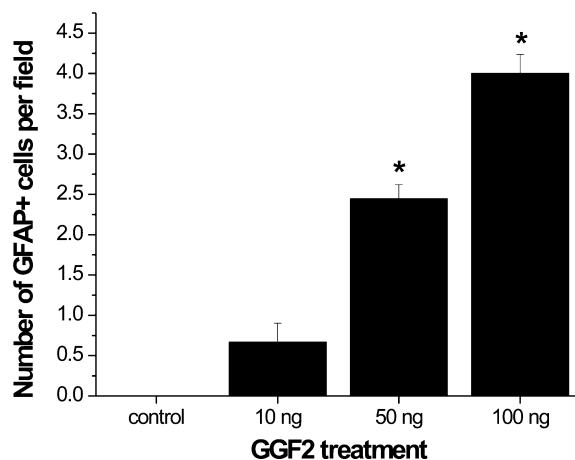

Fig. 10 The number of glial fibrillary acidic protein-positive (GFAP+) cells in serum-free midbrain culture at day *in vitro* (DIV 9). The cultures were treated with three doses of glial growth factor-2 (GGF2) throughout the entire experiment. No GFAP+ cells were present in control groups; however, the number of GFAP+ cells was increased with the higher doses of GGF2; *$p < 0.05$ vs. control.

revealed that GFAP protein levels were significantly higher in 100 ng/mL GGF2-treated cultures vs. those treated with 50 ng mL GGF2 (Fig. 11).
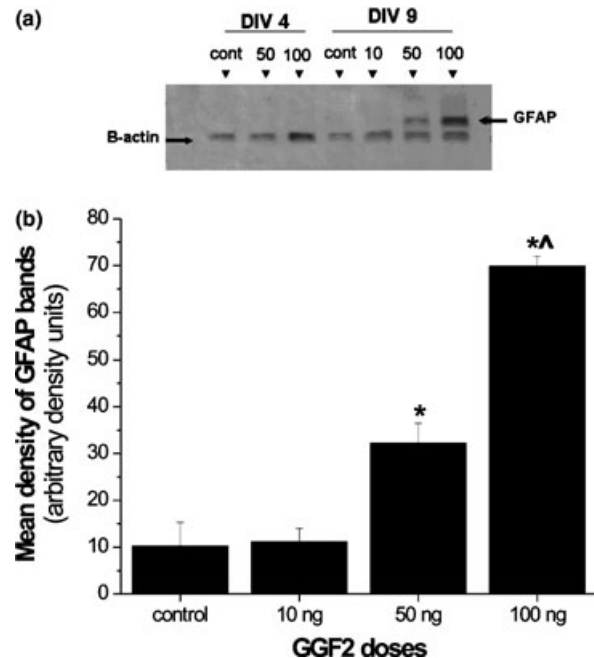

Fig. 11 Western blots for glial fibrillary acidic protein (GFAP) in serum-free midbrain cultures. (a) Consistent with the immunostaining for GFAP in serum-free midbrain cultures, the western blots show no detectable bands in control and glial growth factor-2 (GGF2)-treated cultures at day *in vitro* (DIV) 4. At DIV 9, bands are detected in cultures treated with the higher doses of GGF2 (50 and 100 ng). Quantification of the bands at DIV 9 is shown in (b).

Discussion

In the present study we demonstrate that GGF2 exerts neurotrophic and neuroprotective effects on normal and injured dopaminergic neurons developing in culture. GGF2 significantly promoted dopaminergic neuron survival and increased DA uptake under both serum-free and neurotoxic conditions. Moreover, GGF2 potently enhanced dopaminergic neurite outgrowth. We also present evidence that three NRG receptors are expressed in the primary midbrain cultures and that the number of astrocytes is minimal. Together, these data indicate GGF2 as a novel trophic factor for mesencephalic dopaminergic neurons and suggest that NRGs may represent a novel class of growth factors for use in the treatment of Parkinson's disease.

We first tested the effects of GGF2 in serum-free medium. Serum has been shown to have profound survival effects on cultured DA neurons, and serum deprivation is considered as a mild insult to DA neurons in primary midbrain cell cultures and can eventually result in cell death (Takeshima *et al.* 1994a, 1996). The three doses of GGF2 (10, 50, and 100 ng) used in this study are similar to those previously employed (Bermingham-McDonogh *et al.* 1996; Calaora *et al.* 2001; Goldshmit *et al.* 2001). We found that cultures treated with GGF2 not only exhibited significantly higher numbers of DA

cells, but that neurites on these TH+ cells were more numerous and longer than neurites on the TH+ cells in control cultures at DIV 9; these effects occurred in a dose-dependent manner. We noted that the number of TH+ cells dropped at a similar rate during the first 4 days *in vitro* in both treatment and control cultures. However, during the next 4 days the number of TH+ cells in the control cultures showed a rapid decline, whereas the number of TH+ cells in cultures treated with 50 or 100 ng GGF2 remained relatively stable (Fig. 1). By DIV 9, the number of TH+ cells in GGF2-treated cultures remained significantly higher than the TH+ cells in the control cultures. This observation suggests that GGF2 might have a greater effect on maturating or degenerating dopaminergic neurons, or that the dopaminergic neurons in later culture stages were more sensitive to GGF2. Previous reports indicate that DA cells do not survive well in cultures without any trophic factors or serum supplement (Takeshima *et al*. 1994a, 1996). Our observations also support these results; we noted that TH+ cells show morphological maturation between DIV 2–4 in terms of the size of cell bodies and fiber length and branches. After 4 or 5 days *in vitro*, untreated DA cells started showing degenerating signs, including fiber shrinkage and a reduction in branches, along with rapid decreases in numbers of TH+ cells. By DIV 9 there are only very few TH+ cells left in the control cultures. These morphological changes of TH+ cells in the later culture stages (from DIV 4 to DIV 9) found in the control cultures were significantly attenuated by 50 or 100 ng GGF2 treatment. Despite the decreases in the number of TH+ cells, the GGF2-treated cultures appeared healthy, with long fiber outgrowth and robust branches. These morphological changes become more obvious at higher doses of GGF2 treatment.

The high-affinity DA uptake results further confirmed the above observations. All cultures treated with the three doses of GGF2 exhibited an increase in DA uptake, although only the higher doses (50 and 100 ng) reached statistical significance in comparison to the control cultures. Similar to the TH cell numbers, the DA uptake values increased in a dose-dependent manner. This result is very intriguing because it suggests that GGF2 not only protects the TH cell bodies, but also protects and/or preserves TH neurites and terminals, where most of the DA transporters are located. Preservation of dopaminergic terminals can be critical in terms of normal nigrostriatal DA function. For example, several studies that have evaluated the protective effects of neurotrophic factors against lesion-induced neurodegeneration of the nigrostriatal pathway report negligible improvements in functional recovery when the treatments spare only TH+ cells in the substantia nigra without a concomitant preservation of TH+ terminals in the striatum (Altar *et al*. 1992; Knusel *et al*. 1992; Rosenblad *et al*. 1999, 2000; Kordower *et al*. 2000).

The neuroprotective effect of GGF2 was further tested by our 6-OHDA neurotoxin experiments. Dopaminergic neuron degeneration induced by 6-OHDA in culture is broadly used for testing the neuroprotective effects of DA-specific trophic factors; however, the doses of 6-OHDA and the time of exposure used by different groups are variable (Spina *et al*. 1992a,b; Hou *et al*. 1997; Eggert *et al*. 1999; Kramer *et al*. 1999; Guo *et al*. 2001). In the current study, we exposed midbrain cultures to 50 μM 6-OHDA for 45 min at DIV 4. In control cultures this treatment resulted in a loss of 50% of the TH+ neurons and reduced DA uptake values to 50% of control values. This 6-OHDA treatment regimen is consistent with other neurotoxin studies of primary midbrain cultures in that the number of surviving TH+ neurons is titrated to about 50% of control values prior to experimental manipulation (e.g. Bilsland *et al*. 2002). Treatment of the 6-OHDA-challenged cultures with 100 ng GGF2 completely prevented TH+ cell death and improved DA uptake values to 80% of control values. The 100 ng GGF2 dose is based on our serum deprivation data in which the higher dose of GGF2 was more effective than the lower doses tested. In pilot studies, we noted that higher doses or longer exposure times of 6-OHDA could cause broad and even near complete cell death in midbrain cultures; this observation is consistent with some other reports (Michel and Hefti 1990; Kramer *et al*. 1999). In order to make the culture stable enough to withstand the rather harsh neurotoxin treatment at a later time point, we added 5% serum during the first 24 h after plating, and then switched to the fresh serum-free medium thereafter.

NRG, initially discovered as a glial mitogen, has profound effects on glial proliferation, differentiation, migration and survival. Several studies have reported that mesencephalic glial cells enhance dopaminergic neuron survival *in vitro* (Engele *et al*. 1991; O'Malley *et al*. 1991, 1992; Takeshima *et al*. 1994a,b; Sortwell *et al*. 2000). Although the midbrain cultures used for this study were neuron-enriched, approximately 5% of the undifferentiated cells at the time of plating could potentially develop into glia (Takeshima *et al*. 1996). To determine whether GGF2 promoted mesencephalic glial proliferation, which could raise the possibility that the effects of GGF2 on DA cells were mediated by mesencephalic glia, we examined cultures for the presence of GFAP using immunocytochemical and western blot analyses in serum-free midbrain cultures. At DIV 4, we failed to detect GFAP+ cells in control, 10 ng, or 50 ng GGF2-treated cultures, and only isolated GFAP+ cells were observed in cultures treated with 100 ng GGF2. At DIV 9, the number of GFAP+ cells in 50 and 100 ng GGF2-treated cultures was significantly greater than the number of GFAP+ cells in control cultures and the increase was dose-dependent. However, there were only four to five GFAP+ cells per field (Fig. 11). These results show that even though GGF2 promotes a small degree of mesencephalic glial proliferation, this effect is not very potent, suggesting that the neuroprotective effects are not mediated by glia in these cultures. Consistent with the GFAP

cell number data, western blots for GFAP protein showed the expected 50-kDa band in 50 and 100 ng GGF2-treated culture samples only at DIV 9.

Although GGF2 promotion of astrocyte proliferation was minimal in these cultures, the issue is raised as to the possibility of similar effects upon administration *in vivo*. Moreover, whether such a response may be detrimental or beneficial to the survival of midbrain DA cells in Parkinson's disease is uncertain. On the one hand, reactive astrocytes could be neuroprotective for DA cells in Parkinson's disease due to production of beneficial trophic factors, by scavenging toxic oxidants, and by uptake of excess extracellular glutamate (for reviews, see Vila *et al*. 2001; Mena *et al*. 2002; Teismann *et al*. 2003). On the other hand, reactive astrocytes could have a deleterious role by producing noxious compounds such as reactive oxygen and nitrogen species, pro-inflammatory agents and cytokines (Vila *et al*. 2001; Mena *et al*. 2002; Teismann *et al*. 2003). Thus, future studies of GGF2 treatment in animal models of Parkinson's disease will need to monitor possible responses of reactive glia in the ventral mesencephalon.

Three NRG receptors were detected in the midbrain cultures as early as DIV 1 and expression continued until DIV 9, the endpoint of these experiments. As the cultures used in this study were neuron-enriched and no evidence of detectable GFAP+ cells was found in the control cultures, these data are consistent with the notion that the NRG receptors (ErbB2, ErbB3, and ErbB4) are most likely expressed on neurons in the developing midbrain cultures. Quantitative analysis of western blots in the present study showed there were no significant changes in receptor protein levels at the different time points tested in our culture conditions. As this is a heterogeneous midbrain culture containing different neuronal subpopulations, including a large percentage of GABAergic neurons in addition to the dopaminergic neurons (as well as infrequent glia detected in the cultures treated with GGF2 at later *in vitro* times), it is difficult to draw a conclusion as to which subtype(s) of cells expresses certain NRG receptors or of which ErbB receptor is mainly activated. However, considering the evidence that nigrostriatal dopaminergic neurons *in vivo* express ErbB4 mRNA (Steiner *et al*. 1999; Yurek and Seroogy 2001), and that ErbB4 is the only NRG receptor that can function by itself by forming homodimers, we can speculate that ErbB4 may be the main player in mediating the trophic effects of NRGs on dopaminergic neurons in these midbrain cultures. Consistent with previous studies (Kornblum *et al*. 2000), our *in situ* hybridization data showed that E14 rat ventral mesencephalon expressed robust ErbB4 mRNA. As the primary midbrain cell cultures were derived from E14 ventral mesencephalon, these data also supported our western blot result,s which showed that ErbB4 protein was expressed in the midbrain cell cultures on DIV 1 through DIV 9. This midbrain area also contains a high percentage of dopamin-

ergic neurons during development, and this has been confirmed by our TH mRNA *in situ* hybridization results, as well as by other previous studies (Specht *et al*. 1981; Voorn *et al*. 1988) that showed TH mRNA highly expressed in E14 ventral mesencephalon. The overlapping TH and ErbB4 mRNA labeling results indirectly provide evidence that mesencephalic DA cells expressed the ErbB4 receptor and raise the possibility that the ErbB4 receptor may play important roles in dopaminergic neuron differentiation, maturation and migration during development.

The present results are consonant with previous *in vitro* studies of NRG effects in 'neuronal-like' and neuron-enriched cultures and extend the repertoire of NRG's actions to mesencephalic neurons. In a series of studies using the PC12 neuroblastoma model, Pinkas-Kramarski and colleagues found that NRG, acting via the ErbB4 receptor, promoted neurite extension, increased cellular survival by inhibiting apoptosis induced by serum deprivation, and protected the cells against oxidative stress-induced cell death (Vaskovsky *et al*. 2000; Erlich *et al*. 2001; Goldshmit *et al*. 2001). Our present data in midbrain neuronal cultures are consistent with these trophic effects of NRG in PC12 cells. Relatively few previous studies have examined NRG actions in CNS neuronal cultures. NRG was recently shown to protect cultured neurons derived from embryonic cortex against serum deprivation-induced apoptosis (Li *et al*. 2003). In cultured neonatal cerebellar granule cells, NRG enhanced neurite outgrowth, but did not affect survival of the cells (Rieff *et al*. 1999). Finally, in cultures of embryonic and neonatal retinal cells, many of which are ganglion cells, GGF2 promoted neuronal survival and neurite outgrowth in serum-free cultures, in a dose-dependent manner (Berming-ham-McDonogh *et al*. 1996). It was noted that glia were not present in the retinal cultures, akin to our midbrain serum-free preparations. Results from this latter study are remarkably similar to our present findings in developing mesence-phalic cultures. The trophic effects of NRG on PC12 cells and in the cortical cultures are mediated via the mitogen-activated protein kinase or phosphatidylinositol 3-kinase intracellular signaling pathways (Vaskovsky *et al*. 2000; Erlich *et al*. 2001; Goldshmit *et al*. 2001; Li *et al*. 2003). The signaling mechanisms mediating the present neurotroph-ic and neuroprotective effects of GGF2 on dopaminergic neurons in our midbrain cultures remain to be determined.

In summary, the present data suggest that mesencephalic dopaminergic neurons have the capacity to respond to NRGs during development. This is based on the growth factor's ability to protect DA neurons from 6-OHDA toxic insults, its capacity to reduce the normal attrition of cultured DA neurons, its promotion of neuritic outgrowth of dopaminergic fibers in serum-free culture conditions, and the presence of NRG receptors in the cultures. Thus, these neurotrophic and neuroprotective actions of GGF2 on midbrain dopaminergic cells raise the initial possibility that

GGF2/NRG may have potential usefulness for the treatment of Parkinson's disease. Moreover, it will be valuable in future studies to compare the beneficial effects of NRGs on dopaminergic neurons to those of other more well-characterized neurotrophic factors, in particular glial cell line-derived neurotrophic factor.

Acknowledgements

References

Adlkofer K. and Lai C. (2000) Role of neuregulins in glial cell development. *Glia* **29**, 104–111.

Altar C. A., Boylan C. B., Jackson C., Hershenson S., Miller J., Wiegand S. J., Lindsay R. M. and Hyman C. (1992) Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover in vivo. *Proc. Natl Acad. Sci. USA* **89**, 11 347–11 351.

Apparsundaram S., Schroeter S., Giovanetti E. and Blakely R. D. (1998) Acute regulation of norepinephrine transport. II. PKC-modulated surface expression of human norepinephrine transporter proteins. *J. Pharmacol. Exp. Ther.* **287**, 744–751.

Bermingham-McDonogh O., McCabe K. L. and Reh T. A. (1996) Effects of GGF/neuregulins on neuronal survival and neurite outgrowth correlate with erbB2/neu expression in developing rat retina. *Development* **122**, 1427–1438.

Bermingham-McDonogh O., Xu Y. T., Marchionni M. A. and Scherer S. S. (1997) Neuregulin expression in PNS neurons: isoforms and regulation by target interactions. *Mol. Cell. Neurosci.* **10**, 184–195.

Bilsland J., Roy S., Xanthoudakis S., Nicholson D. W., Han Y., Grimm E., Hefti F. and Harper S. J. (2002) Caspase inhibitors attenuate 1-methyl-4-phenylpyridinium toxicity in primary cultures of mesencephalic dopaminergic neurons. *J. Neurosci.* **22**, 2637–2649.

Blum D., Torch S., Lambeng N., Nissou M., Benabid A. L., Sadoul R. and Verna J. M. (2001) Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog. Neurobiol.* **65**, 135–172.

Burden S. and Yarden Y. (1997) Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron* **18**, 847–855.

Calaora V., Rogister B., Bismuth K., Murray K., Brandt H., Leprince P., Marchionni M. and Dubois-Dalcq M. (2001) Neuregulin signaling regulates neural precursor growth and the generation of oligodendrocytes in vitro. *J. Neurosci.* **21**, 4740–4751.

Cannella B., Pitt D., Marchionni M. and Raine C. S. (1999) Neuregulin and erbB receptor expression in normal and diseased human white matter. *J. Neuroimmunol.* **100**, 233–242.

Canoll P. D., Musacchio J. M., Hardy R., Reynolds R., Marchionni M. A. and Salzer J. L. (1996) GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. *Neuron* **17**, 229–243.

Collier T. J. and Sortwell C. E. (1999) Therapeutic potential of nerve growth factors in Parkinson's disease. *Drugs Aging* **14**, 261–287.

Dong Z., Brennan A., Liu N., Yarden Y., Lefkowitz G., Mirsky R. and Jessen K. R. (1995) Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. *Neuron* **15**, 585–596.

Eggert K., Schlegel J., Oertel W., Wurz C., Krieg J. C. and Vedder H. (1999) Glial cell line-derived neurotrophic factor protects dopaminergic neurons from 6-hydroxydopamine toxicity in vitro. *Neurosci. Lett.* **269**, 178–182.

Engele J., Schubert D. and Bohn M. C. (1991) Conditioned media derived from glial cell lines promote survival and differentiation of dopaminergic neurons in vitro: role of mesencephalic glia. *J. Neurosci. Res.* **30**, 359–371.

Erlich S., Goldshmit Y., Lupowitz Z. and Pinkas-Kramarski R. (2001) ErbB-4 activation inhibits apoptosis in PC12 cells. *Neuroscience* **107**, 353–362.

Falls D. L. (2003) Neuregulins: functions, forms, and signaling strategies. *Exp. Cell Res.* **284**, 14–30.

Gassmann M. and Lemke G. (1997) Neuregulins and neuregulin receptors in neural development. *Curr. Opin. Neurobiol.* **7**, 87–92.

Gerecke K. M., Wyss J. M., Karavanova I., Buonanno A. and Carroll S. L. (2001) ErbB transmembrane tyrosine kinase receptors are differentially expressed throughout the adult rat central nervous system. *J. Comp. Neurol.* **433**, 86–100.

Goldshmit Y., Erlich S. and Pinkas-Kramarski R. (2001) Neuregulin rescues PC12-ErbB4 cells from cell death induced by H(2)O(2). Regulation of reactive oxygen species levels by phosphatidylinositol 3-kinase. *J. Biol. Chem.* **276**, 46 379–46 385.

Grunblatt E., Mandel S. and Youdim M. B. (2000) MPTP and 6-hydroxydopamine-induced neurodegeneration as models for Parkinson's disease: neuroprotective strategies. *J. Neurol.* **247** (suppl. 2), II95–II102.

Guo X., Dawson V. L. and Dawson T. M. (2001) Neuroimmunophilin ligands exert neuroregeneration and neuroprotection in midbrain dopaminergic neurons. *Eur. J. Neurosci.* **13**, 1683–1693.

Hou J. G., Cohen G. and Mytilineou C. (1997) Basic fibroblast growth factor stimulation of glial cells protects dopamine neurons from 6-hydroxydopamine toxicity: involvement of the glutathione system. *J. Neurochem.* **69**, 76–83.

Knusel B., Beck K. D., Winslow J. W., Rosenthal A., Burton L. E., Widmer H. R., Nikolics K. and Hefti F. (1992) Brain-derived neurotrophic factor administration protects basal forebrain cholinergic but not nigral dopaminergic neurons from degenerative changes after axotomy in the adult rat brain. *J. Neurosci.* **12**, 4391–4402.

Kordower J. H., Emborg M. E., Bloch J. *et al.* (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* **290**, 767–773.

Kornblum H. I., Yanni D. S., Easterday M. C. and Seroogy K. B. (2000) Expression of the EGF receptor family members ErbB2, ErbB3, and ErbB4 in germinal zones of the developing brain and in neurosphere cultures containing CNS stem cells. *Dev. Neurosci.* **22**, 16–24.

Kramer B. C., Goldman A. D. and Mytilineou C. (1999) Glial cell line derived neurotrophic factor promotes the recovery of dopamine neurons damaged by 6-hydroxydopamine in vitro. *Brain Res.* **851**, 221–227.

Krane I. M. and Leder P. (1996) NDF/heregulin induces persistence of terminal end buds and adenocarcinomas in the mammary glands of transgenic mice. *Oncogene* **12**, 1781–1788.

Li B. S., Ma W., Jaffe H., Zheng Y., Takahashi S., Zhang L., Kulkarni A. B. and Pant H. C. (2003) Cyclin-dependent kinase-5 is involved in neuregulin-dependent activation of phosphatidylinositol 3-kinase and Akt activity mediating neuronal survival. *J. Biol. Chem.* **278**, 35 702–35 709.

Lindsay R. M., Altar C. A., Cedarbaum J. M., Hyman C. and Wiegand S. J. (1993) The therapeutic potential of neurotrophic factors in the treatment of Parkinson's disease. *Exp. Neurol.* **124**, 103–118.

Marchionni M. A., Cannella B., Hoban C. *et al.* (1999) Neuregulin in neuron/glial interactions in the central nervous system. GGF2 diminishes autoimmune demyelination, promotes oligodendrocyte progenitor expansion, and enhances remyelination. *Adv. Exp. Med. Biol.* **468**, 283–295.

Mena M. A., de Bernardo S., Casarejos M. J., Canals S. and Rodriguez-Martin E. (2002) The role of astroglia on the survival of dopamine neurons. *Mol. Neurobiol.* **25**, 245–263.

Meyer D. and Birchmeier C. (1994) Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development. *Proc. Natl Acad. Sci. USA* **91**, 1064–1068.

Michel P. P. and Hefti F. (1990) Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture. *J. Neurosci. Res.* **26**, 428–435.

Moller J. C., Sautter J. and Kupsch A. (1996) Potential of neurotrophic factors in therapy of Parkinson's disease. *J. Neural Transm. Suppl.* **48**, 103–112.

Numan S. and Seroogy K. B. (1997) Increased expression of trkB mRNA in rat caudate–putamen following 6-OHDA lesions of the nigrostriatal pathway. *Eur. J. Neurosci.* **9**, 489–495.

O'Malley E. K., Black I. B. and Dreyfus C. F. (1991) Local support cells promote survival of substantia nigra dopaminergic neurons in culture. *Exp. Neurol.* **112**, 40–48.

O'Malley E. K., Sieber B. A., Black I. B. and Dreyfus C. F. (1992) Mesencephalic type I astrocytes mediate the survival of substantia nigra dopaminergic neurons in culture. *Brain Res.* **582**, 65–70.

Obeso J. A., Rodriguez-Oroz M. C., Rodriguez M., Lanciego J. L., Artieda J., Gonzalo N. and Olanow C. W. (2000) Pathophysiology of the basal ganglia in Parkinson's disease. *Trends Neurosci.* **23**, S8–S19.

Parker M. W., Chen Y., Hallenbeck J. M. and Ford B. D. (2002) Neuregulin expression after focal stroke in the rat. *Neurosci. Lett.* **334**, 169–172.

Pinkas-Kramarski R., Eilam R., Spiegler O., Lavi S., Liu N., Chang D., Wen D., Schwartz M. and Yarden Y. (1994) Brain neurons and glial cells express Neu differentiation factor/heregulin: a survival factor for astrocytes. *Proc. Natl Acad. Sci. USA* **91**, 9387–9391.

Raabe T. D., Francis A. and DeVries G. H. (1998) Neuregulins in glial cells. *Neurochem. Res.* **23**, 311–318.

Rieff H. I., Raetzman L. T., Sapp D. W., Yeh H. H., Siegel R. E. and Corfas G. (1999) Neuregulin induces GABA(A) receptor subunit expression and neurite outgrowth in cerebellar granule cells. *J. Neurosci.* **19**, 10 757–10 766.

Rio C., Rieff H. I., Qi P., Khurana T. S. and Corfas G. (1997) Neuregulin and erbB receptors play a critical role in neuronal migration. *Neuron* **19**, 39–50.

Rosenblad C., Kirik D., Devaux B., Moffat B., Phillips H. S. and Björklund A. (1999) Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or the lateral ventricle. *Eur. J. Neurosci.* **11**, 1554–1566.

Rosenblad C., Kirik D. and Björklund A. (2000) Sequential administration of GDNF into the substantia nigra and striatum promotes dopamine neuron survival and axonal sprouting but not striatal reinnervation or functional recovery in the partial 6-OHDA lesion model. *Exp. Neurol.* **161**, 503–516.

Sortwell C. E., Daley B. F., Pitzer M. R., McGuire S. O., Sladek J. R. Jr and Collier T. J. (2000) Oligodendrocyte-type 2 astrocyte-derived trophic factors increase survival of developing dopamine neurons through the inhibition of apoptotic cell death. *J. Comp. Neurol.* **426**, 143–153.

Specht L. A., Pickel V. M., Joh T. H. and Reis D. J. (1981) Light-microscopic immunocytochemical localization of tyrosine hydroxylase in prenatal rat brain. II. Late ontogeny. *J. Comp. Neurol.* **199**, 255–276.

Spina M. B., Hyman C., Squinto S. and Lindsay R. M. (1992a) Brain-derived neurotrophic factor protects dopaminergic cells from 6-hydroxydopamine toxicity. *Ann. N. Y. Acad. Sci.* **648**, 348–350.

Spina M. B., Squinto S. P., Miller J., Lindsay R. M. and Hyman C. (1992b) Brain-derived neurotrophic factor protects dopamine neurons against 6-hydroxydopamine and *N*-methyl-4-phenylpyridinium ion toxicity: involvement of the glutathione system. *J. Neurochem.* **59**, 99–106.

Stefansson H., Sigurdsson E., Steinthorsdottir V. *et al.* (2002) Neuregulin 1 and susceptibility to schizophrenia. *Am. J. Hum. Genet.* **71**, 877–892.

Steiner H., Blum M., Kitai S. T. and Fedi P. (1999) Differential expression of ErbB3 and ErbB4 neuregulin receptors in dopamine neurons and forebrain areas of the adult rat. *Exp. Neurol.* **159**, 494–503.

Takeshima T., Johnston J. M. and Commissiong J. W. (1994a) Mesencephalic type 1 astrocytes rescue dopaminergic neurons from death induced by serum deprivation. *J. Neurosci.* **14**, 4769–4779.

Takeshima T., Johnston J. M. and Commissiong J. W. (1994b) Oligodendrocyte-type-2 astrocyte (O-2A) progenitors increase the survival of rat mesencephalic, dopaminergic neurons from death induced by serum deprivation. *Neurosci. Lett.* **166**, 178–182.

Takeshima T., Shimoda K., Johnston J. M. and Commissiong J. W. (1996) Standardized methods to bioassay neurotrophic factors for dopaminergic neurons. *J. Neurosci. Meth.* **67**, 27–41.

Teismann P., Tieu K., Cohen O., Choi D. K., Wu du C., Marks D., Vila M., Jackson-Lewis V. and Przedborski S. (2003) Pathogenic role of glial cells in Parkinson's disease. *Mov. Disord.* **18**, 121–129.

Tokita Y., Keino H., Matsui F., Aono S., Ishiguro H., Higashiyama S. and Oohira A. (2001) Regulation of neuregulin expression in the injured rat brain and cultured astrocytes. *J. Neurosci.* **21**, 1257–1264.

Vaskovsky A., Lupowitz Z., Erlich S. and Pinkas-Kramarski R. (2000) ErbB-4 activation promotes neurite outgrowth in PC12 cells. *J. Neurochem.* **74**, 979–987.

Vila M., Jackson-Lewis V., Guegan C., Wu D. C., Teismann P., Choi D. K., Tieu K. and Przedborski S. (2001) The role of glial cells in Parkinson's disease. *Curr. Opin. Neurol.* **14**, 483–489.

Villegas R., Villegas G. M., Longart M., Hernandez M., Maqueira B., Buonanno A., Garcia R. and Castillo C. (2000) Neuregulin found in cultured-sciatic nerve conditioned medium causes neuronal differentiation of PC12 cells. *Brain Res.* **852**, 305–318.

Voorn P., Kalsbeek A., Jorritsma-Byham B. and Groenewegen H. J. (1988) The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neuroscience* **25**, 857–887.

Yarden Y. and Sliwkowski M. X. (2001) Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* **2**, 127–137.

Yurek D. M. and Seroogy K. B. (2001) Neurotrophic factor protection of dopaminergic neurons, in *Neurobiology of the Neurotrophins* (I. Mocchetti, ed.), pp. 355–397. F. P. Graham Publishing, Johnson City, TN, USA.

Zhang L., Dhillon H. S., Barron S., Hicks R. R., Prasad R. M. and Seroogy K. B. (2000) Effects of chronic ethanol administration on expression of BDNF and trkB mRNAs in rat hippocampus after experimental brain injury. *Brain Res. Mol. Brain Res.* **79**, 174–179.

Zhao J. J. and Lemke G. (1998) Selective disruption of neuregulin-1 function in vertebrate embryos using ribozyme-tRNA transgenes. *Development* **125**, 1899–1907.

BRAIN RESEARCH

Short communication

Supranigral injection of neuregulin1-β induces striatal dopamine overflow

David M. Yurek[a,*], Lixin Zhang[a,b], Anita Fletcher-Turner[a], Kim B. Seroogy[c]

[a]Department of Surgery/Neurosurgery, University of Kentucky College of Medicine, Health Sciences Research Building, Lexington, KY 40536-0305, United States
[b]Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Lexington KY 40536, United States
[c]Department of Neurology, University of Cincinnati, Cincinnati, OH 45267, United States

Abstract

Previous studies have provided anatomical evidence that the functional neuregulin receptor, ErbB4, is present within the ventral midbrain where it is co-localized to dopamine neurons of the substantia nigra and ventral tegmental area. In this study, we provide evidence that neuregulin1-β (a.k.a. heregulin1-β), a neuregulin-1 gene isoform that preferentially binds to and activates the ErbB4 receptor, evokes an almost immediate overflow of striatal dopamine when injected into a region just dorsal to the ipsilateral substantia nigra. These data are indicative that neuregulins can modulate the activity of mesostriatal dopaminergic neurons.
© 2004 Elsevier B.V. All rights reserved.

Neuregulins are a family of structurally related growth and differentiation factors that have been shown to play important roles in neural differentiation, migration, and survival, particularly in the early developing nervous system (for recent reviews, see Refs. [2,7]). Functions of neuregulins in the mature central nervous system (CNS), however, remain largely undefined. Neuregulin ligand and neuregulin receptor (ErbB2, ErbB3, and ErbB4) transcripts and protein are broadly but discretely distributed throughout many areas of adult rat brain, including cerebral cortex, hippocampus, amygdala, thalamic reticular nucleus, mesencephalon, cerebellum, and medulla [4,5,8,11,15]. The ErbB tyrosine kinase receptors are differentially expressed in neurons and glia, with ErbB4 more prevalent in neurons, and ErbB3 primarily expressed in glia [15]. The widespread expression of neuregulin (NRG) receptors in the adult rat brain suggests possible roles for NRG in the mature nervous system.

Previous studies have reported that ErbB4 is expressed within a substantial population of cells within the rodent substantia nigra (SN) and ventral tegmental area (VTA) [15,16]. Moreover, neurotoxic lesioning studies have shown a decrease of ErbB4 expression following degeneration of the dopaminergic nigrostriatal pathway [15,16], indicating that the dopamine cells express the neuregulin receptor. We have also recently determined that NRG exhibits trophic and protective effects upon dopaminergic neurons in primary midbrain cultures [18]. Given these data, it is conceivable adult ventral mesencephalic neurons are responsive to neuregulins in vivo. The purpose of this study was to determine whether or not an acute intracerebral injection of an ErbB4 receptor ligand, neuregulin1-β (NRG1-β) [2,7], into the ventral midbrain alters the neurochemical activity of dopaminergic neurons in the nigrostriatal pathway.

* Corresponding author. Tel.: +1 859 257 8219; fax: +1 859 323 6343.
E-mail address: David.Yurek@uky.edu (D.M. Yurek).

Adult male Sprague–Dawley rats (225–275 g body weight) were purchased from Harlan Farm (Indianapolis, IN), and housed under 12:12-h light/dark conditions with free access to food and water. All experimental protocols involving animals were approved by the University of Kentucky Institutional Animal Use and Care Committee and are in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals and the Society for Neuroscience Guidelines for the Use of Animals in Neuroscience Research.

Microdialysis was used to measure extracellular striatal dopamine levels both before and after a supranigral infusion of NRG1-β (extracellular domain, R&D Systems); this analysis was performed using a technique described in an earlier study [17]. Briefly, each animal was anesthetized with a halothane–air mixture (1.0–1.5% halothane at 1.5 l/min), placed into a stereotaxic frame, and maintained under halothane anesthesia for the duration of the microdialysis sampling period. Body temperature was maintained at 37 °C using a heating pad (CMA 150 Temperature Controller, Carneige Medicin). Concentric dialysis probes (3.0 mm membranes, 0.5 mm diameter, CMA/12, Carneige Medicin) were used for all intracerebral dialysis studies. Dialysis probes were stereotactically lowered into each striatum (bilateral, +0.5 mm AP, ±2.5 mm ML; relative to bregma with the skull in a flat position) until the tip of the dialysis probe was 6.5 mm below the dura. The inlet of the dialysis probes were connected to a microinjection pump (CMA 100, Carneige Medicin) and perfusate (147 mM NaCl, 1.0 mM $CaCl_2$, 3.9 mM KCl, pH=6.0) was continuously pumped into the probe at a flow rate of 2.0 μl/min. The first sample period began 1.0 h after the probe was stereotactically lowered into the brain. Striatal perfusates were collected every 25 min in microcentrifuge tubes containing 10 μl of 0.1 M perchloric acid used as a preservative. After two baseline samples were collected, 10 μg NRG1-β (dissolved in 2.0 μl PBS) or 2.0 μl PBS (vehicle control) was stereotactically injected 1.0 mm above the left substantia nigra at the following coordinates: AP=−5.4 mm, ML=+2.2 mm, DV=−7.5 mm (relative to bregma with the skull in a flat position and measured from the top of the skull) using a 10 μl Hamilton microsyringe. The injection rate was 0.5 μl/min for 4 min and at the completion of each injection the needle was left in place for 2 min and then slowly withdrawn. Each sample was subsequently stored at −80 °C. Sample size for each group: NRG1-β (*n*=12), PBS control (*n*=3). Samples were assayed for levels of dopamine, DOPAC and HVA using HPLC with electrochemical detection as described [17]. Compounds were separated on a 150×3 mm MD-150 column (ESA). Mobile phase (pH=3.0, 75 mM sodium phosphate, 0.1 mM EDTA, 3.0 mM octyl sodium sulfate, and 10% acetonitrile) was pumped at a rate of 0.6 ml/min. The HPLC system was coupled to dual-coulometric detectors (Model 5014B, ESA) with a pre-conditioning electrode set at −175 mV and the detection electrode set at 150 mV.

Dialysate values were corrected for probe recoveries at 37 °C and are reported relative to 100% recovery. Probe recoveries were in the range of 9–15%±1.4% for dopamine and 16–20%±1.9% for DOPAC and HVA. Statistical analysis was performed using a two-way repeated-measures analysis of variance (ANOVA).

Extracellular dopamine levels were measured using the intracerebral microdialysis technique in order to investigate if neuregulin affected dopamine overflow in the normal rat striatum. Fig. 1 summarizes the effect of a unilateral supranigral injection of NRG1-β on dopamine overflow in the ipsilateral and contralateral striatum. The first basal sample was collected 1.0 h after the microdialysis probes were lowered into the brain tissue. NRG1-β was injected just after the second baseline sample was collected (time 0). All NRG1-β treated animals showed significant increases in dopamine overflow in the ipsilateral striatum at the various time points following the injection of NRG1-β; among 12 animals that received NRG1-β injections, 11 showed an immediate (25–50 min) increase in dopamine overflow (Fig. 1). Statistical analysis revealed significant increases in striatal dopamine levels in the ipsilateral striatum at time points 25 and 50 min. On the other hand, supranigral infusion of PBS had no affect on dopamine levels in the
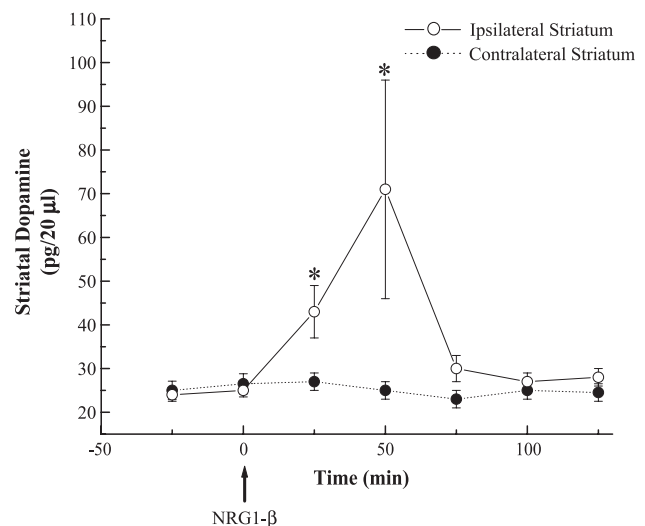

Fig. 1. Dopamine overflow in the striatum before and after an supranigral infusion of NRG1-β (*n*=12). Microdialysis samples were collected every 25 min. Two basal samples were collected (at time points −25 and 0) and the injection of NRG1-β (10 μg) was made immediately after the second basal sample was collected, indicated by the arrow; injection coordinates: AP −5.4, ML +2.2, and DV −7.5 using bregma as a reference point and the skull in a flat position. Open circles indicate mean dopamine values (±S.E.M.) for samples obtained from the striatum ipsilateral to the injection of NRG1-β and black circles indicate mean dopamine values (±S.E.M.) for samples obtained from the striatum contralateral to the injection of NRG1-β. Statistical analysis of dopamine values revealed a significant SIDE (ipsilateral, contralateral)×TIME interaction [$F_{(6,167)}$=4.15, $p < 0.001$]. Simple main effect comparisons were made for means of each treatment at each time point. Mean values for dopamine at time points 25 and 50 for the ipsilateral striatum were significantly greater than the mean dopamine values for the contralateral striatum at the same time points, *$p < 0.05$.
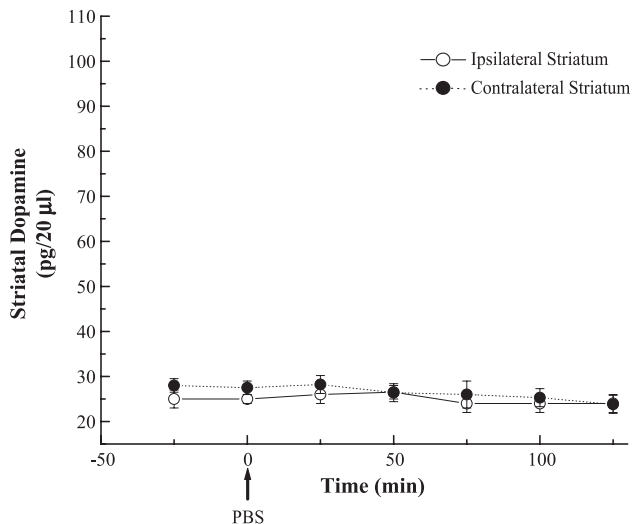
Fig. 2. Dopamine overflow in the striatum before and after an intranigral infusion of PBS (n=3). Two basal samples were collected (at time points −25 and 0) and the injection of PBS (2.0 μl) was made immediately after the second basal sample was collected, indicated by the arrow; injection coordinates: AP −5.4, ML +2.2, and DV −7.5 using bregma as a reference point and the skull in a flat position. Open circles indicate mean dopamine values (±S.E.M.) for samples obtained from the striatum ipsilateral to the injection of PBS and black circles indicate mean dopamine values (±S.E.M.) for samples obtained from the striatum contralateral to the injection of PBS. Statistical analysis revealed no significant difference between dopamine levels measured in the ipsilateral or contralateral striatum at any time points [$F(1,41)$=0.85, p>0.05].

ipsilateral striatum when compared to values in the contralateral striatum or to baseline values (Fig. 2). NRG1-β treatment did not have any effect on DOPAC levels [$F(1,167)$=1.05, p>0.05] or HVA levels [$F(1,167)$=0.325, p>0.05] in either the ipsilateral or contralateral striatum. As additional controls, we injected heregulin-α, an alternatively spliced product of the neuregulin-1 gene that is similar in structure to NRG1-β and not found in the CNS, or the neurotoxin 6-hydroxydopamine into the same site we injected NRG1-β and neither one of these molecules evoked striatal dopamine overflow during the same time period NRG1-β evoked striatal dopamine overflow (data not shown).

These results provide evidence that an acute supranigral injection of NRG1-β evokes an immediate and significant increase in dopamine overflow in the ipsilateral striatum. This result indicates mature dopaminergic neurons in the SN can respond to NRG1-β. The present study is among the first to analyze the actions of NRG1-β following its administration into the mature brain. To our knowledge, only two previous studies have employed direct infusion of NRG1-β into the rat brain. Using electrophysiological techniques, Roysommuti et al. [13] found that intrahippo-campal administration of NRG1-β differentially modulated synaptic transmission in two hippocampal circuits. Penderis et al. [12] infused the neuregulin glial growth factor-2 (GGF2) into the caudal cerebellar peduncle in a rat model of demyelination, but found no effect on remyelination and did

not report any other effects of the growth factor. We now show that a single intraparenchymal injection of NRG1-β just above the SN pars compacta alters neurochemical parameters of the dopaminergic nigrostriatal pathway in the normal rat.

The functional neuregulin receptor ErbB4 is widely expressed in adult rat mesencephalon and, more specifically, by midbrain dopaminergic neurons themselves. Therefore, the present response is likely mediated by the ErbB4 receptor on dopaminergic neurons since NRG1-β is known to bind and activate ErbB4 [2,3,7]. However, we cannot exclude the possibility that this effect is mediated indirectly via ErbB3 or ErbB4 receptors expressed on glia or another neuronal population in the ventral midbrain. Stimulation of such neural cells to secrete certain known or unknown neurotrophic factors could sequentially affect the dopaminergic neurons. Our recent data, however, indicate that midbrain GABAergic neurons rarely express the ErbB4 receptor [14]. Administration of certain other dopamine-associated trophic factors has been shown to elicit neurochemical changes in the nigrostriatal system. For example, acute infusion of brain-derived neurotrophic factor (BDNF) into a supranigral region can alter dopamine turnover rate in the ipsilateral striatum [1]. Similarly, an intranigral infusion of glial cell line-derived neurotrophic factor (GDNF) increases basal dopamine overflow as well as dopamine overflow evoked by either amphetamine of potassium treatment [9]. We cannot rule out the possibility NRG1-β's effect on dopamine overflow occurs via indirect mechanisms that affect the activity of other factors, e.g., BDNF and GDNF. Nevertheless, these data clearly show that NRG1-β influences the activity of midbrain nigrostriatal dopaminergic neurons; however, it remains to be determined which cellular mechanisms translate the activation of mesencephalic neuregulin receptors into increased dopamine overflow in the striatum. Overall, these findings may have potential application to the treatment of Parkinson's disease, which is characterized neuropathologically by the degeneration of midbrain dopaminergic neurons and subsequent depletion of striatal dopamine [6,10]. Factors that can stimulate the secretion of dopamine in the striatum have the potential to overcome the lack of dopaminergic function in individuals with Parkinson's disease.

Acknowledgements

References

[1] C.A. Altar, C. Boylan, C. Jackson, S. Hershenson, J. Miller, S.J. Wiegand, R.M. Lindsay, C. Hyman, Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover in vivo, Proc. Natl. Acad. Sci. 89 (1992) 11347–11351.

[2] A. Buonanno, G.D. Fischbach, Neuregulin and ErbB receptor signaling pathways in the nervous system, Curr. Opin. Neurobiol. 11 (2001) 287–296.

[3] G. Carpenter, ErbB-4: mechanism of action and biology, Exp. Cell Res. 284 (2003) 66–77.

[4] M.S. Chen, O. Bermingham-McDonogh, F.T. Danehy Jr., C. Nolan, S.S. Scherer, J. Lucas, D. Gwynne, M.A. Marchionni, Expression of multiple neuregulin transcripts in postnatal rat brains, J. Comp. Neurol. 349 (1994) 389–400.

[5] G. Corfas, K.M. Rosen, H. Aratake, R. Krauss, G.D. Fischbach, Differential expression of ARIA isoforms in the rat brain, Neuron 14 (1995) 103–115.

[6] S. Fahn, Description of Parkinson's disease as a clinical syndrome, Ann. N.Y. Acad. Sci. 991 (2003) 1–14.

[7] D.L. Falls, Neuregulins: functions, forms, and signaling strategies, Exp. Cell Res. 284 (2003) 14–30.

[8] K.M. Gerecke, J.M. Wyss, I. Karavanova, A. Buonanno, S.L. Carroll, ErbB transmembrane tyrosine kinase receptors are differentially expressed throughout the adult rat central nervous system, J. Comp. Neurol. 433 (2001) 86–100.

[9] M.A. Hebert, G.A. Gerhardt, Behavioral and neurochemical effects of intranigral administration of glial cell line-derived neurotrophic factor on aged Fischer 344 rats, J. Pharmacol. Exp. Ther. 282 (1997) 282–760.

[10] K.A. Jellinger, The pathology of Parkinson's disease, Adv. Neurol. 86 (2001) 55–72.

[11] D. Meyer, C. Birchmeier, Multiple essential functions of neuregulin in development, Nature 378 (1995) 386–390.

[12] J. Penderis, R.H. Woodruff, A. Lakatos, W.W. Li, M.D. Dunning, C. Zhao, M. Marchionni, R.J. Franklin, Increasing local levels of neuregulin (glial growth factor-2) by direct infusion into areas of demyelination does not alter remyelination in the rat CNS, Eur. J. Neurosci. 18 (2003) 2253–2264.

[13] S. Roysommuti, S.L. Carroll, J.M. Wyss, Neuregulin-1beta modulates in vivo entorhinal–hippocampal synaptic transmission in adult rats, Neuroscience 121 (2003) 779–785.

[14] K.B. Seroogy, J.P. Herman, K.H. Lundgren, ErbB4 neuregulin receptor and GAD mRNAs are extensively colocalized in forebrain, but not ventral midbrain GABAergic neurons, Soc. Neurosci. Abstr. (2004) 38.5.

[15] H. Steiner, M. Blum, S.T. Kitai, P. Fedi, Differential expression of erbB3 and erbB4 neuregulin receptors in dopamine neurons and forebrain areas of the adult rat, Exp. Neurol. 494 (1999) 494–503.

[16] D.M. Yurek, K.B. Seroogy, Neurotrophic factor protection of dopaminergic neurons, in: I. Mocchetti (Ed.), Neurobiology of the Neurotrophins, F.P. Graham Publishing Co., Johnson City, TN, 2001.

[17] D.M. Yurek, S.B. Hipkens, M.A. Hebert, D.M. Gash, G.A. Gerhardt, Age-related decline in striatal dopamine release and motoric function in Brown Norway/Fischer 344 hybrid rats, Brain Res. 791 (1998) 246–256.

[18] L. Zhang, A. Fletcher-Turner, K.H. Lundgren, M. Marchionni, D.M. Yurek, K.B. Seroogy, Neurotrophic and neuroprotective effects of the neuregulin glial growth factor-2 on dopaminergic neurons in rat primary midbrain cultures, J. Neurochem. (2004) (in press).

195

Neuregulins

KIM B. SEROOGY AND LIXIN ZHANG

ABSTRACT

Neuregulins are pleiotrophic growth factors that influence cell survival, proliferation, differentiation and organogenesis throughout the body. Their effects are mediated via interactions with the ErbB family of transmembrane receptor protein tyrosine kinases and the subsequent activation of downstream intracellular signaling events. This chapter focuses on the expression and emerging neurotrophic, neuroprotective, and neuromodulatory roles of neuregulins in the central nervous system.

DISCOVERY AND STRUCTURE

Neuregulins (NRGs) are a family of structurally related signaling proteins that bind to receptor tyrosine kinases of the ErbB family and mediate a myriad of cellular functions, including survival, proliferation, and differentiation in both neuronal and nonneuronal systems. Discovered independently over a decade ago by several different groups, these peptide growth factors were originally described as neu differentiation factor (NDF), heregulins, glial growth factors (GGFs), acetyl-choline receptor–inducing activity (ARIA), and sensory and motor neuron-derived factor (SMDF) (for review, see [17]). It is now known that all these NRG proteins are encoded by the same gene, named NRG-1. Four genes (termed NRG-1, NRG-2, NRG-3, and NRG-4) have been identified that encode NRGs in vertebrates, and the best characterized of these is the NRG-1 gene [1, 3, 18]. Recently, the entire human NRG-1 gene was sequenced [56].

At least 15 isoforms are generated by multiple pro-motor use and alternative splicing of the NRG-1 gene, including the NDFs [48, 63], heregulins [31], GGFs [25, 42], ARIA [18], and SMDF [27]. Several isoforms are also produced from the NRG-2 gene [51, 65]. The most important portion of the NRG molecule is the epidermal growth factor (EGF)-like domain shared by all the isoforms because it alone is sufficient for receptor binding and the stimulation of intracellular signaling pathways; hence, the EGF domain is both necessary and sufficient for biological activity. This domain contains approximately 50 amino acids and is characterized by three pairs of cysteines that are important for its tertiary structure and biological function. The alternate splice variant at this domain gives rise to α and β isoforms [3, 6]. Three types of NRG-1 (type I, II, and III) are also divided based on differences of the extracellular domains; types I and II have an immunoglobulinlike (IgG-like) domain followed by either a glycosylation-rich region (type I) or a GGF-specific (kringle) domain (type II), whereas type III has a cysteine-rich domain (CRD). In addition, all three types of NRG-1 have both transmembrane forms and secreted forms depending on whether the isoform is initially synthesized as a transmembrane or nonmembrane protein [3, 17].

DISTRIBUTION AND PROCESSING

Neuregulin-1 transcripts are broadly but discretely expressed throughout the nervous system during development and in adulthood [8, 10, 35, 37, 44], with highest levels of expression in brain-stem motor and sensory nuclei, spinal cord motor neurons, and sensory ganglia. Neuregulin-1 mRNAs are also found in the cerebral cortex, diencephalon, hippocampus, basal forebrain, substantia nigra and cerebellum, and the proliferative forebrain subventricular zone. Neuregulin-2 and -3 mRNAs are also expressed in the brain, but exhibit distinct expression patterns from NRG-1, with NRG-2 primarily restricted to the olfactory bulb, cerebellum,

1419

and hippocampal dentate gyrus in the adult, whereas NRG-3 expression appears widespread [41]. Both NRG-1 and NRG-2 mRNAs, as opposed to NRG-3 mRNA, are developmentally regulated, with the highest levels of expression found neonatally. Neuregulin-4 transcripts have not been detected in the central nervous system [26]. For the most part, immunohistochemical demonstrations of NRG-1 and NRG-2 proteins correspond fairly well with their mRNA localization in both the rodent and human brain [35, 37, 41].

The proteolytic processing of NRG proproteins has recently been reviewed [17]. Briefly, most (but not all) NRGs in the nervous system are initially synthesized as single transmembrane proproteins with an extracellular N-terminal ectodomain (containing the biologically active EGF domain), a juxtamembrane stalk region, the transmembrane domain, and the intracellular cytoplasmic tail. A bioactive ectodomain fragment is generated and released via the metalloprotease-mediated cleavage of the proprotein in the stalk region. Other NRGs are produced directly as soluble secreted proteins (e.g., GGF2). The type III NRG proproteins have two transmemembrane domains, including an N-terminal region that loops back through the membrane resulting in an intracellular N-terminus, in addition to the intracellular C-terminus. Proteolytic cleavage at the stalk region results in an anchored N-terminal fragment (with the bioactive EGF-like region); further juxtamembrane proteolytic processing may release the fragment [17]. Another mode of NRG processing recently described is backsignaling, in which, on NRG type III/ErbB binding, the intracellular C-terminal domain is proteolytically released into the cytoplasm where it subsequently travels to the nucleus and modulates gene expression [2]. The ultimate result of these various avenues of NRG isoform processing is that NRGs garner the capacity to signal via autocrine, paracrine, juxtacrine, and possibly intracrine mechanisms.

RECEPTORS

Neuregulins interact directly or indirectly with members of a subfamily of receptor tyrosine kinase proteins called ErbB receptors. The four members of the ErbB family include the EGF receptor (also known as ErbB1; the human homolog is human EGF receptor 1, HER1), ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. All family members have in common an extracellular ligand-binding N-terminal domain, a single transmembrane portion, a cytoplasmic tyrosine kinase domain, and a cytoplasmic C-terminal domain containing autophophorylation sites. On ligand binding, the receptors form homo- or heterodimers, which consequently stimulate the intrinsic tyrosine kinase activity of

the receptors and triggers autophosphorylation of specific tyrosine residues within the cytoplasmic domain (for recent reviews see [5, 9, 66]). These phosphorylated residues serve as docking sites for signaling molecules involved in the regulation of intracellular signaling cascades, mainly through the stimulation of the Ras-Raf-ERK mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-3 kinase (PI3-K) pathway, the PLCγ-PKC pathway, and the JAK-STAT pathway [9]. An alternative signaling mechanism for ErbB4 upon ligand binding includes presenilin-dependent intramembrane proteolysis by γ-secretase to release an intracellular ErbB4 C-terminal fragment that translocates to the nucleus to regulate gene expression [38, 45]. The ErbB receptors form a complex hierarchical network based on their relative affinity for the ligands, intrinsic tyrosine kinase activities, ability to recruit specific signaling molecules, and cellular distribution [61]. This network is critical to the ErbB signaling pathway because ErbB2, which exhibits the highest degree of kinase activity, has no known direct ligands and ErbB3 is kinase-defective; therefore, neither of them can function by homodimerization. However, by heterodimerizing with other ErbB receptors, they can form potent signaling assemblies (i.e., ErbB2/ErbB3, ErbB3/ErbB4, etc.) and ErbB2 has been demonstrated to be the most potent partner to any ErbB family member. ErbB4 itself contains moderate kinase activity; therefore, its presence alone is sufficient for biological activity, and it can function via both homo- and heterodimerization.

Neuregulins can directly bind to only the ErbB3 and ErbB4 receptors, which can then heterodimerize with ErbB2 (an orphan receptor) and ErbB1 to activate and/or potentiate subsequent signaling activity. All four NRG gene products can bind ErbB4, whereas only NRG-1 and NRG-2 proteins can bind ErbB3 [5, 9, 66]. ErbB4 is also noteworthy for the fact that multiple isoforms of the receptor may exist based on sequence alterations in the extracellular stalk region and in the cytoplasmic C-terminal domain; the latter variation can influence PI3-K function [33]. Interestingly, some members of the EGF ligand family, including betacellulin, epiregulin, and heparin-binding EGF-like growth factor (HB-EGF), can bind and activate the ErbB4 receptor [5, 30]. Thus, not only are there complex interactions among the ErbB receptor family members after NRG binding but the possibility of receptor cross-activation with select EGF family ligands must be taken into consideration.

ErbB receptor mRNAs are differentially expressed throughout the nervous system during development, both spatially and temporally, and are continuously expressed throughout adulthood. ErbB2, ErbB3, and ErbB4 transcripts and protein are broadly expressed

throughout all areas of the adult rat brain and spinal cord, including the cortex, amygdala, hippocampus, medial habenula, reticular thalamic nucleus, substantia nigra pars compacta, ventral tegmental area, cerebellum, pons, and medulla [8, 10, 19, 23, 36, 44, 49, 57]. These three receptors are differentially expressed in neurons and glia. In general, ErbB4 is more prevalent in the neurons and ErbB3 in the glia in white matter tracts, whereas ErbB2 is present in both neural cell types at lower density. Notably, ErbB4 expression defines a large subset of GABAergic interneurons in forebrain regions [19, 55] and dopaminergic neurons in the ventral midbrain [57, 67, 69], suggesting some specific neurotransmitter-related functions for NRG-ErbB4 signaling in the brain. Moreover, the widespread expression of ErbB receptors in the adult brain points to numerous roles for NRG in the mature nervous system.

NRG-2 versus NRG-1

The overall domain structure of NRG-2 gene products resembles NRG-1 proteins (such as GGF2) and include EGF-like, Ig, transmembrane, and cytoplasmic sequences. As in NRG-1, there appear to be two versions (α and β) of the EGF domain, which have distinct activities. Recent evidence, however, suggests important distinctions between NRG-1 and NRG-2, leading to the possibility of differing functions on central nervous system (CNS) neurons (see [59] for review). Studies have shown that coupling to downstream signaling molecules, including differential phosphorylation of select tyrosine residues on ErbB4, differs among various NRG-1, -2, -3, and -4 ligands [28, 29, 58]. Differential signaling and bioactivities of NRG-1 versus NRG-2 ligands have been observed in various cell lines [12, 50]. Two consistent findings from these studies were that NRG-2β was more potent than other NRG-2 and NRG-1 isoforms and that some specific NRG receptor (ErbB1-4) combinations appeared to be more responsive than others to isoforms of NRG-2. For example, cells expressing ErbB4 or the ErbB4-ErbB1 combination showed a particularly robust response to NRG-2 proteins. These results could shed light on potential cellular targets for NRG-2 throughout the CNS.

Within the rodent CNS, intriguing differences between NRG-1 and NRG-2 have recently been identified [41]. For example, with respect to mRNA expression, the pattern of NRG-2 is essentially distinct from and sometimes complementary to sites mapped for NRG-1 expression. Thus, the products of these two different genes could have distinct functions in different brain circuits. The temporal patterns of expression in the brain also differ between the two genes. NRG-1 mRNA expression is relatively high perinatally, followed by downregulation during maturation into adulthood.

In contrast, NRG-2 mRNA expression in the brain is the opposite, low prenatally and high in adulthood [41]. It thus has been hypothesized that NRG-1 might function preferentially in early prenatal and perinatal developmental events, whereas NRG-2 may be the preferred NRG ligand in the mature brain [59]. Finally, the subcellular distribution of the two NRGs differ in that NRG-2 is targeted to dendrites, whereas NRG-1 is mainly localized to axonal and somatic compartments [41], suggesting different functional roles for the ligands in the establishment, maintenance, and plasticity of synaptic connections. Taken together, the specific and distinct molecular and biochemical interactions of the two NRG ligands with their receptors, as well as their differential spatial, temporal, and subcellular expression patterns in the brain, point to some unique aspects of NRG-2 vs NRG-1 neural signaling.

BIOLOGICAL ACTIONS

First recognized as glial cell mitogens, the NRGs are now believed to play essential roles in cell survival, migration, and differentiation in both neural and non-neural cells. Outside the nervous system, NRGs are essential, for example, for proper development of the heart, mammary gland, and skin. In terms of dysfunction, NRG-ErbB signaling is associated with many different cancers, including those of the breast, lung, bladder, ovary, and brain [5, 9, 17, 30]. The remainder of this review focuses on the emerging neuroactive functions of NRGs and their receptors in the nervous system, and particularly in the CNS.

The most well-characterized functions of NRGs associated with the nervous system include roles in glial cell proliferation, maturation, and survival; neuromuscular acetylcholine receptor induction; and early neuronal migration and survival (see [1, 3, 17, 43] for reviews). Moreover, NRGs may have important neurotrophic functions in the brain in addition to their well-known actions in the peripheral nervous system [1, 3]. The analysis of mutant (knockout) mice deficient in either NRG-1, ErbB2, ErbB3, or ErbB4 reveals a severe reduction in several neural crest-derived cell populations, including Schwann cells, neural crest-derived cranial sensory neurons, and sympathetic neurons, and demonstrate critical roles for the receptors and ligand in aspects of hindbrain development (e.g., [15, 24, 53]). Mice deficient in heregulin (heterozygous mutants) exhibited hyperactivity in multiple behavioral tasks, suggesting abnormalities in motor performance [21]. The findings in cerebellar slices and cultures that NRG increases the expression of an NMDA receptor subunit (NR2C) and the GABA$_A$ receptor β2 subunit demonstrate that NRGs can regulate neurotransmitter

receptors in the brain, as well as at the neuromuscular junction [46, 47, 52]. A trophic function is also suggested by the demonstration that NRGs enhance neurite outgrowth from cultured cerebellar granule cells [52], hippocampal neurons [22], and PC12 cells [62]. The potential role of NRGs and their receptors in activity-dependent neuronal mechanisms is underscored by the demonstration that several forms of activity (seizures; forced locomotion; long-term potentiation, LTP) induce an upregulation of NRG and ErbB4 expression in hippocampal, neocortical, and amygdaloid neurons [14]. In hippocampal slices, NRG inhibited the induction of LTP [32]. In hippocampal cultures, NRGs increased the α7 subunit of nicotinic acetylcholine receptors and enhanced excitory synaptic transmission in GABAergic interneurons [39]. Finally, in the adult rat in vivo, NRGs have recently been found to differentially modulate synaptic transmission in two hippocampal circuits [54]. These results, combined with results showing that ErbB4 is enriched in brain postsynaptic densities and interacts functionally with PSD-95 in hippocampal synapses [20, 31], suggest a possible modulatory role for neuregulins in adult brain synaptic plasticity. The proliferation of neuronal progenitor cells in the brain is also regulated by NRGs [40]. Taken together, these findings provide a strong basis to postulate neurotrophic and neuromodulatory functions for NRGs and their receptors in brain neuronal systems.

Neuregulins Are Neurotrophic for Nigrostriatal Dopaminergic Neurons

ErbB4 mRNA is expressed within a substantial population of dopaminergic neurons within the substantia nigra and ventral tegmental area of both rodent and monkey [23, 57, 67] (K. B. Seroogy, unpublished). These results suggest that ventral mesencephalic neurons could be responsive to the trophic effects of NRGs. Indeed, as determined by in vivo microdialysis, a single supranigral injection of the NRGs heregulin-β1 or GGF2 induced a substantial increase in dopamine overflow in the ipsilateral striatum [68]. This was among the first in vivo demonstrations of NRG actions in the brain and provided initial evidence that NRGs can augment dopaminergic nigrostriatal function.

Consistant with these findings, recent in vitro results indicate substantial neurotrophic and neuroprotective roles for GGF2 in primary cell cultures derived from fetal rat mesencephalon [69]. The effects of GGF2 were tested on developing dopaminergic neurons in the midbrain cultures under both serum-free and 6-OHDA-challenged conditions. It was found that GGF2 significantly promoted dopaminergic neuron survival, increased dopamine uptake, and enhanced dopaminergic fiber outgrowth under both conditions compared with controls. Glial cells are absent or extremely rare in these serum-free midbrain cultures, suggesting that the neurotrophic effects of GGF2 on the dopaminergic cells are direct, and not mediated via glia. Notably, in separate preliminary experiments in midbrain cultures, the NRG-2 protein NRG-2β also enhanced dopamine neuron survival and process outgrowth, and protected the dopamine cells against 6-OHDA-induced degeneration (D. M. Yurek and K. B. Seroogy, unpublished).

Neuregulins and Neurological Disorders

Increasing evidence of roles for NRG-ErbB signaling in the etiology and treatment of several neurological disorders is rapidly accumulating. In a chronic relapsing experimental model of multiple sclerosis, administration of GGF2 resulted in significant beneficial effects, including a reduction in inflammation and demyelination and enhanced remyelination [4]. Supported by the well-known trophic effects of NRGs on oligodendrocytes and the suppression of activated microglial responses by GGF2 [13], these findings raise the possibility of NRGs as a treatment for demyelinating disorders. In animal models of stroke, both NRG and ErbB receptors are upregulated in the peri-infarct region in both neurons and macrophages/microglia. Treatment with NRG-1 in these paradigms reduced infarct volume, blocked secondary neuronal degeneration and pro-inflammatory responses, and improved functional outcome, indicating a neuroprotective role for NRGs in ischemic brain injury [64]. A potential role for NRGs in brain trauma is suggested by the upregulation of NRG ligands and receptors in neurons, astrocytes, and microglia in animal models of experimental brain injury [16, 60]. Similarly, the expression of NRG-1 within neuritic plaques and of both NRG-1 and ErbB4 within reactive astrocytes and microglia surrounding the neuritic plaques in human Alzheimer's disease brain raises the possibility of NRG-ErbB mechanisms in this debilitating neurodegenerative disorder [7]. Much excitement has been generated within the realm of psychiatric disease with the identification of the NRG-1 gene as a probable susceptibility gene for schizophrenia [56]. The intriguing possibility that defects in NRG/ErbB signaling contribute to the molecular or cellular basis of schizophrenia has been recently reviewed [11]. Finally, the recent demonstration that NRG-1 can cross the blood–brain barrier in a receptor-mediated manner [34] holds promise in terms of the potential therapeutic delivery of NRGs for the treatment of CNS disorders.

Acknowlegments

02-1-0174). We thank Kerstin Lundgren for invaluable assistance.

References

[1] Adlkofer K, Lai C. Role of neuregulins in glial cell development. Glia 2000;29:104–11.

[2] Bao J, Wolpowitz D, Role LW, Talmage DA. Back signaling by the Nrg-1 intracellular domain. J Cell Biol 2003;161:1133–41.

[3] Buonanno A, Fischbach GD. Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr Opin Neurobiol 2001;11:287–96.

[4] Cannella B, Hoban CJ, Gao Y, Garcia-Arenas R, Lawson D, Marchionni M et al. The neuregulin, glial growth factor 2, diminishes autoimmune demyelination and enhances remyelination in a chronic relapsing model of multiple sclerosis. Proc Natl Acad Sci USA 1998;95:10100–5.

[5] Carpenter G. ErbB-4: mechanism of action and biology. Exp Cell Res 2003;284:66–77.

[6] Carraway KL, 3rd, Burden SJ. Neuregulins and their receptors. Curr Opin Neurobiol 1995;5:606–12.

[7] Chaudhury AR, Gerecke KM, Wyss JM, Morgan DG, Gordon MN, Carroll SL. Neuregulin-1 and erbB4 immunoreactivity is associated with neuritic plaques in Alzheimer disease brain and in a transgenic model of Alzheimer disease. J Neuropathol Exp Neurol 2003;62:42–54.

[8] Chen MS, Bermingham-McDonogh O, Danehy FT, Nolan C, Scherer SS, Lucas J et al. Expression of multiple neuregulin transcripts in postnatal rat brains. J Comp Neurol 1994;349: 389–400.

[9] Citri A, Skaria KB, Yarden Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. Exp Cell Res 2003;284:54–65.

[10] Corfas G, Rosen KM, Aratake H, Krauss R, Fischbach GD. Differential expression of ARIA isoforms in the rat brain. Neuron 1995;14:103–15.

[11] Corfas G, Roy K, Buxbaum JD. Neuregilin 1-erbB signaling and the molecular/cellular basis of schizophrenia. 2004;7: 575–80.

[12] Crovello CS, Lai C, Cantley LC, Carraway KL III. Differential signaling by the epidermal growth factor-like growth factors neuregulin-1 and neuregulin-2. J Biol Chem 1998;273: 26954–61.

[13] Dimayuga FO, Ding Q, Keller JN, Marchionni MA, Seroogy KB, Bruce-Keller AJ. The neuregulin GGF2 attenuates free radical release from activated microglial cells. J Neuroimmunol 2003; 136:67–74.

[14] Eilam R, Pinkas-Kramarski R, Ratzkin BJ, Segal M, Yarden Y. Activity-dependent regulation of Neu differentiation factor/neuregulin expression in rat brain. Proc Natl Acad Sci USA 1998;95:1888–93.

[15] Erickson SL, O'Shea KS, Ghaboosi N, Loverro L, Frabtz G, Bauer M et al. ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2- and heregulin-deficient mice. Development 1997;124:4999–5011.

[16] Erlich S, Shohami E, Pinkas-Kramarski R. Closed head injury induces up-regulation of ErbB-4 receptor at the site of injury. Mol Cell Neurosci 2000;16:597–608.

[17] Falls DL. Neuregulins: functions, forms, and signaling strategies. Exp Cell Res 2003;284:14–30.

[18] Falls DL, Rosen KM, Corfas G, Lane WS, Fischbach GD. ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell 1993;72:801–15.

[19] Fox IJ, Kornblum HI. Developmental profile of ErbB receptors in murine central nervous system: implications for functional interactions. J Neurosci Res 2005;79:584–97.

[20] Garcia RA, Vasudevan K, Buonanno A. The neuregulin receptor ErbB-4 interacts with PDZ-containing proteins at neuronal synapses. Proc Natl Acad Sci USA 2000;97:3596–601.

[21] Gerlai R, Pisacane P, Erickson S. Heregulin, but not ErbB2 or ErbB3, heterozygous mutant mice exhibit hyperactivity in multiple behavioral tasks. Behav Brain Res 2000;109:219–27.

[22] Gerecke KM, Wyss JM, Carroll SL. Neuregulin-1β induces neurite extension and arborization in cultured hippocampal neurons. Mol Cell Neurosci 2004;27:379–93.

[23] Gerecke KM, Wyss JM, Karavanova I, Buonanno A, Carroll SL. ErbB transmembrane tyrosine kinase receptors are differentially expressed throughout the adult rat central nervous system. J Comp Neurol 2001;433:86–100.

[24] Golding JP, Trainor P, Krumlauf R, Gassman M. Defects in path-finding by cranial neural crest cells in mice lacking the neuregulin receptor ErbB4. Nature Cell Biol 2000;2:103–9.

[25] Goodearl AD, Davis JB, Mistry K, Minghetti L, Otsu M, Waterfield MD et al. Purification of multiple forms of glial growth factor. J Biol Chem 1993;268:18095–102.

[26] Harari D, Tzahar E, Romano J, Shelly M, Pierce JH, Andrews GC et al. Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. Oncogene 1999;18:2681–9.

[27] Ho WH, Armanini MP, Nuijens A, Phillips HS, Osheroff PL. Sensory and motor neuron-derived factor. A novel heregulin variant highly expressed in sensory and motor neurons. J Biol Chem 1995;270:14523–32.

[28] Hobbs SS, Cameron EM, Hammer RP, Le ATD, Gallo RM, Blommel EN et al. Five carboxyl-terminal residues of neuregulin2 are critical for stimulation of signaling by the ErbB4 receptor tyrosine kinase. Oncogene 2004;23:883–93.

[29] Hobbs SS, Coffing SL, Le ATD, Cameron EM, Williams EE, Andrew M et al. Neuregulin isoforms exhibit distinct patterns of ErbB family receptor activation. Oncogene 2002;21:8442–52.

[30] Holbro T, Hynes NE. ErbB receptors: directing key signaling networks throughout life. Ann Rev Pharmacol Toxicol 2004;44: 195–217.

[31] Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW et al. Identification of heregulin, a specific activator of p185erbB2. Science 1992;256:1205–10.

[32] Huang YZ, Won S, Ali DW, Wang Q, Tanowitz M, Du QS et al. Regulation of neuregulin signaling by PSD-95 interacting with ErbB4 at CNS synapses. Neuron 2000;26:443–55.

[33] Juntilla TT, Sundvall M, Määtä JA, Elenius K. ErbB4 and its isoforms. Selective regulation of growth factor responses by naturally occurring receptor variants. Trends Cardiovasc Med 2000;10:304–8.

[34] Kastin AJ, Akerstrom V, Pan W. Neuregulin-1-beta1 enters brain and spinal cord by receptor-mediated transport. J Neurochem 2004;88:965–70.

[35] Kerber G, Streif R, Schwaiger F-W, Kreutberg GW, Hager G. Neuregulin-1 isoforms are differentially expressed in the intact and regenerating adult rat nervous system. J Mol Neurosci 2003;21:149–65.

[36] Kornblum HI, Yanni DS, Easterday MC, Seroogy KB. Expression of the EGF receptor family members ErbB2, ErbB3, and ErbB4 in germinal zones of the developing brain and in neurosphere cultures containing CNS stem cells. Dev Neurosci 2000;22: 16–24.

[37] Law AJ, Shannon Weickert C, Hyde TM, Kleinman JE, Harrison PJ. Neuregulin-1 (NRG-1) mRNA and protein in the adult human brain. Neuroscience 2004;127:125–36.

[38] Lee HJ, Jung KM, Huang YZ, Bennett LB, Lee JS, Mei L et al. Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4. J Biol Chem 2002;277:6318–23.

[39] Liu Y, Ford B, Mann MA, Fischbach GD. Neuregulins increase α7 nicotinic acetylcholine receptors and enhance excitatory

synaptic transmission in GABAergic interneurons of the hippocampus. J Neurosci 2001;21:5660–9.

[40] Liu Y, Ford B, Mann MA, Fischbach GD. Neuregulin-1 increases the proliferation of neuronal progenitors from embryonic neural stem cells. Dev Biol 2005;283:437–45.

[41] Longart M, Liu Y, Karavanova I, Buonanno A. Neuregulin-2 is developmentally regulated and targeted to dendrites of central neurons. J Comp Neurol 2004;472:156–72.

[42] Marchionni MA, Goodearl AD, Chen MS, Bermingham-McDonogh O, Kirk C, Hendricks M et al. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature 1993;362:312–8.

[43] Marchionni MA, Grinspan JB, Canoll PD, Mahanthappa NK, Salzer JL, Scherer SS. Neuregulins as potential neuroprotective agents. Ann NY Acad Sci 1997;825:348–65.

[44] Meyer D, Birchmeier C. Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development. Proc Natl Acad Sci USA 1994;91:1064–68.

[45] Ni CY, Murphy MP, Golde TE, Carpenter G. gamma-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science 2001;294:2179–81.

[46] Ozaki M, Sasner M, Yano R, Lu HS, Buonanno A. Neuregulin-β induces expression of an NMDA-receptor subunit. Nature 1997;390:691–4.

[47] Ozaki M, Tohyama K, Kishida H, Buonanno A, Yano R, Hashikawa T. Roles of neuregulin in synaptogenesis between mossy fibers and cerebellar granule cells. J Neurosc. Res 2000;59:612–23.

[48] Peles E, Bacus SS, Koski RA, Lu HS, Wen D, Ogden SG et al. Isolation of the neu/HER-2 stimulatory ligand: a 44kD glycoprotein that induces differentiation of mammary tumor cells. Cell 1992;69:205–16.

[49] Pinkas-Kramarski R, Eilam R, Alroy I, Levkowitz G, Lonai P, Yarden Y. Differential expression of NDF/neuregulin receptors ErbB-3 and ErbB-4 and involvement in inhibition of neuronal differentiation. Oncogene 1997;15:2803–15.

[50] Pinkas-Kramarski R, Shelly M, Guarino BC, Wang LM, Lyass L, Alroy I et al. ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network. Mol Cell Biol 1998;18:6090–101.

[51] Ponomareva ON, Ma H, Dakour R, Raabe TD, Lai C, Rimer M. Stimulation of acetylcholine receptor transcription by neuregulin-2 requires an N-box response element and is regulated by alternative splicing. Neuroscience 2005;134:495–503.

[52] Rieff HI, Raetzman LT, Sapp DW, Yeh HH, Siegel RE, Corfas G. Neuregulin induces GABA(A) receptor subunit expression and neurite outgrowth in cerebellar granule cells. J Neurosci 1999;19:10757–66.

[53] Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmieir C. Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature 1997;389:725–30.

[54] Roysommuti S, Carroll SL, Wyss JM. Neuregulin-1β modulates in vivo entorhinal-hippocampal synaptic transmission in adult rats. Neuroscience 2003;121:779–85.

[55] Seroogy KB, Herman JP, Lundgren KH. ErbB4 neuregulin receptor and GAD mRNAs are extensively colocalized in forebrain, but not ventral midbrain, GABAergic neurons. Soc Neurosci Abst 2004; #38.5.

[56] Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S et al. Neuregulin 1 and susceptibility to schizophrenia. Am J Hum Genet 2002;71:877–92.

[57] Steiner H, Blum M, Kitai ST, Fedi P. Differential expression of ErbB3 and ErbB4 neuregulin receptors in dopamine neurons and forebrain areas of the adult rat. Exp Neurol 1999;159:494–503.

[58] Sweeney C, Lai C, Riese DJ II, Diamonti J, Cantley LC, Carraway KL III. Ligand discrimination in signaling through an ErbB4 receptor homodimer. J Biol Chem 2000;275:19803–7.

[59] Talmage DA, Role LW. Multiple personalities of neuregulin gene family members. J Comp Neurol 2004;472:134–9.

[60] Tokita Y, Keino H, Matsui F, aono S, Ishiguro H, Higashiyama S et al. Regulation of neuregulin expression in the injured rat brain and cultured astrocytes. J Neurosci 2001;21:1257–64.

[61] Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S et al. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol 1996;16:5276–87.

[62] Vaskovsky A, Lupowitz Z, Erlich S, Pinkas-Kramarski R. ErbB-4 activation promotes neurite outgrowth in PC12 cells. J Neurochem 2000;74:979–87.

[63] Wen D, Peles E, Cupples R, Suggs SV, Bacus SS, Luo Y et al. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell 1992;69:559–72.

[64] Xu Z, Croslan DR, Harris AE, Ford GD, Ford BD. Extended therapeutic window and functional recovery after intraarterial administration of neuregulin-1 after focal ischemic stroke. J Cereb Blood Flow Metab 2005;••:••–••.

[65] Yamada K, Ichino N, Nishii K, Sawada H, Higashiyama S, Ishiguro H et al. Characterization of the human NTAK gene structure and distribution of the isoforms for rat NTAK mRNA. Gene 2000;255:15–24.

[66] Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127–37.

[67] Yurek DM, Seroogy KB. Neurotrophic factor protection of dopaminergic neurons. In: Mochetti I, editor. Neurobiology of the neurotrophins, New York: Sulburger & Graham Publishing; 2001, p 355–97.

[68] Yurek DM, Zhang L, Fletcher-Turner A, Seroogy KB. Supranigral injection of neuregulin1-β induces striatal dopamine overflow. Brain Res 2004;1028:116–9.

[69] Zhang L, Fletcher-Turner A, Lundgren KH, Marchionni M, Yurek DM, Seroogy KB. Neurotrophic and neuroprotective effects of the neuregulin glial growth factor-2 on dopaminergic neurons in rat primary midbrain cultures. J Neurochem 2004;91:1358–68.

1